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A STUDY ON THE HYPERGLYCAEMIC FACTOR IN THE URINE OF PSYCHOTICS.

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MEDUNA and Vaichulis (1948) have shown that an extract of urine, prepared by adsorption upon kaolin at an acid pH followed by elution in alkaline solution, contains a factor which raises the blood glucose level if injected into the peritoneum of a rabbit. This activity is—according to these authors—greater in extracts of the urine of patients with schizophrenia than in those of normal controls.

When this work was repeated for the examination of its clinical specificity, we were able to confirm the presence of a hyperglycaemic factor in some cases, but we were puzzled by the fact that the rise in blood glucose seemed to be due to at least two different agents. The following study is concerned with the investigation of these two agents. It was carried out with the urine of patients who were known consistently to excrete substantial quantities of active material.

The work on the clinical validity of Meduna's assertion will be continued, and reported on in a later communication.

TECHNIQUE.

Our technique was similar to that used by Meduna. Kaolin, 5 g., was added to each litre of urine and the pH adjusted to 4·5 with acetic acid. The mixture was shaken for one hour and stored overnight in the refrigerator. On the next day the mixture was re-shaken for one hour and filtered through filter-paper. The residue was re-suspended in 100 ml. 0·6 N ammonia, shaken for 30 minutes and centrifuged at 3000 r.p.m. for 15 minutes. The supernatant fluid was poured into 400 ml. acetone and the resultant precipitate sedimented in the centrifuge, dried *in vacuo* over sulphuric acid and stored *in vacuo* in the refrigerator. The dried material was re-suspended in 10 ml. distilled water, shaken for 15 minutes, and injected into the peritoneum of a rabbit. Animals of approximately 2 kg. weight were used for all experiments. In all cases the pH of the injected solution was between 6·8 and 7·2. The blood glucose level of the rabbit was determined before the injection, and at intervals of 10, 20, 30, 40, 50 minutes, 1, 2, 3 and 4 hours thereafter.

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RESULTS.

Possibility of Two Factors.

The individual blood glucose response curves differed. When the maximum response was relatively small this was usually reached within one hour, much earlier than in those cases where a higher maximum was reached 2 to 3 hours after the injection. This agrees with Meduna's results in his normal controls—he frequently obtained some hyperglycaemia one hour after the injection with a subsequent return to normal.

When a much greater degree of hyperglycaemia was observed this early rise in blood glucose was also present, but the rise was sustained for a longer period and a higher peak was reached in a period of 2 to 3 hours. In a small number of cases a rapid initial rise in blood glucose was followed by a period of little or no increase, again followed, after a period of approximately one hour, by another rise which reached a peak in approximately 3 hours.

These observations suggested that two hyperglycaemic factors were involved, one a rapidly acting factor of low intensity, present in those cases giving small but early peak figures, but both being present in those cases where after an initial rise a greater degree of hyperglycaemia was observed after a longer period.

Attempted Separation of Two Factors.

When the dried material obtained from urine was suspended in distilled water a considerable quantity of solid material remained undissolved. Prolonged centrifugation of the suspension produced an opalescent supernatant fluid which, when injected into a rabbit, produced a peak hyperglycaemia similar to that of the whole suspension, but a less marked early hyperglycaemia. The sediment was re-suspended in distilled water and injected into another rabbit. Hyperglycaemia again resulted, but in this case the early hyperglycaemia was of the same order as that of the whole suspension, but the degree of later activity was much less. Repetition of this experiment produced similar results. They are summarized in Table I. They show that while there had been some separation of the two factors, it had not been complete.

TABLE I.—*The Rise in Blood Glucose (mg./100 ml.) above the Fasting Level at Intervals of 30 Minutes, 1, 2 and 3 Hours after the Injection of (1) Total Suspension, (2) Supernatant Fluid after Centrifugation, and (3) the Sediment Re-suspended in Distilled Water.*

Experiment.	Total suspension (1).				Supernatant (2).				Suspended sediment (3).			
	30 mins.	1 hr.	2 hrs.	3 hrs.	30 mins.	1 hr.	2 hrs.	3 hrs.	30 mins.	1 hr.	2 hrs.	3 hrs.
1	40	72	130	215	27	41	135	220	45	75	62	15
2	60	120	200	170	20	68	195	180	65	100	56	4
3	36	45	95	73	22	32	90	63	35	42	17	3
4	44	45	101	132	11	23	96	135	41	43	21	7
5	71	91	123	164	14	31	117	159	67	81	42	11

The procedure outlined above was now taken further, and the sediment obtained by the centrifugation of the original suspension was re-suspended, shaken and re-sedimented three times, a total of four preparations being obtained. These fluids were injected into rabbits and the hyperglycaemic effect observed. A typical experimental result is shown in Table II, where it is seen that the maximum rise in blood glucose is produced by the injection of the first supernatant fluid in a period of 3 hours. There is little difference in the activity of the other three preparations, which reached their maximum in a much shorter period.

Consideration of the substances likely to be isolated from urine by the technique of adsorption and elution employed suggested that urates and complexes of amino-acids would probably be the substances present in the greatest quantity.

TABLE II.—*The Rise in Blood Glucose (mg./100 ml.) after the Injection of Fractions Obtained by the Repeated Suspension, Centrifugation and Resuspension of Dried Material from Urine.*

Time after injection.	1st supernatant.	2nd supernatant.	3rd supernatant.	Resuspended sediment.
Fasting .	90	92	88	96
30 minutes	126	131	106	147
1 hour .	196	172	118	179
2 hours .	294	152	109	124
3 hours .	307	117	93	99

Uric acid and amino-acid nitrogen concentrations were determined in the preparations before their injection. In Table III these concentrations are related to the results of injection into the peritoneum of rabbits. The table is divided into sections according to the maximum degree of hyperglycaemia produced by each injection. The columns show successively the maximum rise in blood glucose, the uric acid and amino-acid nitrogen content of the fluids injected, the rise in blood glucose 30 minutes after the injection, and the time of the maximum rise. The mean values for each section from Table III are also shown for greater clarity in Table IV.

Consideration of these tables shows a number of points :

(*a*) In the first four sections, represented by the maximum rise in blood glucose of 1–25, 26–50, 51–75 and 76–100, there is a fairly constant proportionality between the rise in the blood glucose level and the uric acid content of the injected fluid. In the three higher groups this proportionality is no longer present.

(*b*) In the first four groups the proportionality between the rise in blood glucose and the amino-acid nitrogen content of the injected material is less constant.

(*c*) In the two lowest groups, with increases in blood glucose up to 50 mg./100 ml., the level of blood glucose reached 30 minutes after the injection of the substance represents a significantly greater proportion of the maximum level than in the five higher sections, and the time taken to reach the maximum level is shorter.

These results strongly support the suggestion that there are two hyperglycaemic factors : the first, the weaker, more rapidly acting factor is probably uric

acid or its salts; the second, the stronger, more slowly acting, is associated with the amino-acid fraction.

The deductions from the results of Table IV were tested experimentally by

TABLE III.—*The Relationship of the Uric Acid and Amino-Acid Nitrogen Contents (mg.) of an Extract to the Rise in Blood Glucose (mg./100 ml.) produced by it.*

Blood glucose (max. rise).	Uric acid.	Amino-acid N.	Increase in blood glucose at 30 min.	Time of max. blood glucose (min.).
1–25				
5	1·3	1·1	0	45
24	1·2	4·1	24	30
20	0·8	2·3	20	30
21	0·4	1·3	21	30
8	2·7	1·1	8	30
6	1·2	0·4	6	30
2	0·2	0·9	0	180
6	0·7	0·4	6	30
0	0·3	0·5	0	..
11	1·1	0·5	11	30
23	2·4	2·5	23	30
13	0·9	0·3	13	30
15	3·2	0·7	15	30
8	1·7	*nil*	8	30
7	0·3	0·1	0	180
2	0·1	*nil*	0	180
Mean 11	1·2	1·1	10	57
26–50				
31	0·8	1·3	29	60
49	8·5	1·7	0	180
47	4·6	0·9	2	180
40	7·2	1·2	0	120
28	2·1	2·1	0	60
27	3·1	0·9	18	60
50	4·9	2·6	46	60
30	2·7	1·1	24	60
26	4·8	1·6	20	60
31	4·4	0·7	23	60
30	3·7	1·1	18	60
32	4·0	1·7	24	60
32	4·5	2·1	30	60
39	4·4	1·2	39	30
30	4·6	0·2	30	30
33	4·4	0·9	29	60
34	5·3	2·1	34	60
42	4·2	0·2	42	60
Mean 35	4·3	1·3	23	73

TABLE III—(*cont.*).

Blood glucose (max. rise).	Uric acid.	Amino-acid N.	Increase in blood glucose at 30 min.	Time of max. blood glucose (min.).
51–75				
75	12·5	2·7	17	120
74	6·4	1·1	35	60
67	1·7	6·0	49	45
73	4·1	*nil*	41	60
59	9·9	2·6	0	180
53	16·1	3·7	0	180
63	3·1	3·9	7	120
56	5·4	1·2	46	60
Mean 66	6·7	2·7	24	103
76–100				
77	6·8	5·5	56	45
86	7·2	3·2	21	120
80	8·2	0·9	56	60
86	7·3	0·9	22	120
80	8·2	3·1	39	60
83	7·1	0·9	51	60
78	7·9	4·4	59	60
79	7·2	3·2	48	60
83	7·9	3·1	50	60
81	7·2	0·3	36	60
Mean 81	7·5	2·5	44	71
101–150				
101	7·9	12·7	90	60
105	5·6	11·6	60	60
108	4·2	9·4	20	60
116	8·3	8·6	89	120
118	8·8	2·3	38	180
128	6·1	12·9	56	180
139	2·5	9·1	0	180
Mean 116	5·5	9·5	50	120
151–200				
162	0·9	7·0	81	180
188	8·3	13·4	72	180
175	2·7	10·1	45	180
152	0·4	12·7	48	180
166	1·7	12·2	64	120
157	2·9	10·9	72	180
162	7·9	7·1	71	120
158	3·2	10·9	77	180
Mean 165	3·5	10·5	66	165

TABLE III—(*cont.*).

Blood glucose (max. rise).	Uric acid.	Amino-acid N.	Increase in blood glucose at 30 min.	Time of max. blood glucose (min.).
Above 200				
217	3·4	13·1	36	180
240	2·4	13·2	50	180
223	4·2	16·3	71	120
206	3·3	13·1	77	180
203	2·2	9·2	72	180
207	4·7	18·9	47	120
206	11·8	10·6	69	120
215	3·2	9·6	95	120
Mean 216	4·4	13·0	65	150

TABLE IV.—*Summary of Mean Values of Table III.*

Mean peak blood glucose.	Mean uric acid in injection (mg.).	Mean amino-acid N in injection (mg.).	Mean blood glucose at 30 min.	Blood glucose at 30 min. as percentage of peak.	Time of peak.	Ratios. BG/UA.	Ratios. BG/AA.
(1–25) 11	1·2	1·1	10	75 (90)	57	0·11	0·10
(26–50) 35	4·3	1·3	23	67	73	0·12	0·04
(51–75) 66	6·7	2·7	24	37	103	0·10	0·04
(76–100) 81	7·5	2·5	44	54	71	0·09	0·03
(101–150) 116	5·5	9·5	50	45	120	0·05	0·08
(151–200) 165	3·5	10·5	66	46	165	0·02	0·06
(200 +) 216	4·4	13·0	65	30	150	0·02	0·06

the injection of amounts of sodium urate, dissolved in 10 ml. distilled water, corresponding to 5, 10 and 15 mg. of uric acid. Sodium urate was used after preliminary experiments had shown that suspensions of uric acid had little effect. The effect of the injection of these quantities of sodium urate was, in 15 experiments, to raise the blood glucose of the rabbit by a mean of 33, 43 and 51 mg./100 ml. respectively. These rises occurred within a period of one hour, after which there was a return to normal.

Further experiments were made in which quantities of amino-acetic acid, glutamic acid, *p*-aminobenzoic acid, aminopropionic acid and aminoisocaproic acid each equivalent to 10 mg. amino-acid nitrogen were injected into rabbits. Only very small and irregular rises in blood glucose were observed.

DISCUSSION.

There can be little doubt that the hyperglycaemic factor found by Meduna in the urine of psychotic patients consists of two agents if the extract of urine is prepared as suggested by him. The analyses set out in Table III coupled with the results of injection of sodium urate strongly suggest that the first agent consists of the salts of uric acid.

The identity of the second agent, slower in action, but more powerful, is still unknown, but its protein nature is suggested by (*a*) its precipitation and adsorp-

tion upon Kaolin at a specific pH, and (*b*) by the approximation of the degree of activity of an extract to its amino-acid nitrogen concentration.

Moreover, Meduna (1950) has proposed, as a working hypothesis, identity or some relation of the anti-insulin factor he discovered to the diabetogenic factor of the anterior pituitary which, according to Price, Cori and Colowick (1945) blocks hexokinase and prevents phosphorylation of glucose. Weil-Malherbe (1950) has found this reaction inhibited by the plasma of diabetics. There is, therefore, a suggestion that the second agent is hormonal in origin, which would further support the supposition of its protein-like structure.

SUMMARY.

(1) The presence of a hyperglycaemic agent in the urine of certain psychotics has been confirmed.

(2) It has been shown that this agent consists of two fractions.

(3) The first fraction, a weaker, more quickly acting agent, has been shown to consist of the salts of uric acid.

(4) It has been shown that the activity of the stronger, more slowly acting agent is associated with the amino-acid content of the extract.

(5) The identity of this second factor is discussed.

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THE REACTION OF THE ADRENAL CORTEX TO ADRENALINE STRESS AND IDENTIFICATION OF ENDOGENOUS LIBERATED SUGAR-ACTIVE CORTICOID BY A HISTOLOGICAL BIO-ASSAY TECHNIQUE.

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CHROMAFFIN tumours (phaeochromocytoma) are usually associated with paroxysmal hypertension which results from disintegration of the phaeochromocyte cell and liberation of an adrenaline-like substance into the thin walled sinusoidal spaces (Blacklock, Ferguson, Mack, Shafar and Symington, 1947). Many cases, however, are recorded in which the basal blood pressure is persistently elevated, although superimposed paroxysmal attacks may be noted. The cause of this persistent hypertension is not known and, in the hope of throwing some light on the subject, attempts were made to simulate the action of chromaffin tumours in rats by injections of adrenaline. Solutions of 1:1000 adrenaline hydrochloride and 1:100 adrenaline ascorbate in gradually increasing amounts were used, but no evidence of persistent hypertension was found in any of the 80 rats used.

During the course of the investigation the adrenals of one of the animals, which died during a severe adrenaline reaction, were treated with 1 per cent osmic acid solution and showed osmophil-positive lipoids in the widely dilated medullary sinusoids ; apparently in the process of entering the medulla from the cortex (Fig. 3 and 4). Examination of these glands specially fixed for chromaffin tissue (Schmorl, 1928 ; Sevki, 1934) gave negative results for adrenaline when stained by the Giemsa method, thus providing further proof of the cortical origin of the osmophil material. This finding in an animal dying 5 hours after a severe adrenaline reaction suggested that it might be of interest to investigate a series of animals by this means to determine the nature of the lipoid secretion.

Although many excellent histo-chemical methods are available for examination of the adrenal cortex, they alone seemed of limited value in the present problems, and thus a combined histological bio-assay test was used to determine whether or not sugar-active corticoid was present in the material liberated from the adrenal cortex following adrenaline injection. This test is based on the ability of sugar-active corticoid to mobilize blood sugar and increase the glycogen content of the liver of adrenalectomized or starving animals.

METHODS OF INVESTIGATION.

Four groups of animals were taken and, to obviate the histological variations of sex and age which are known to occur in the adrenal glands, adult male albino rats only were used.

Group 1 consisted of 14 rats which varied from 255 to 270 g., in weight. They received initial injections of 30μg. (0·5 minims) 1:1000 adrenaline hydrochloride

(B.D.H.) at one day intervals for 15 to 20 days, when the dose was increased to 60 μg. (1 minim), then 120 μg. The injections were increased in this manner over a period of 110 days until the animals had become adapted to 240 μg. (4 minims) of solution. They were not upset until 180 μg. of adrenaline were being injected, when they became dyspnoeic, respirations being rapid and tachycardia marked. There was erection of the hair and coldness of the extremities and tail. The animals occasionally lay prone and sometimes moved restlessly about the cage. The attack started 10 to 15 minutes after the injection and the rats had recovered in 2 hours. This reaction was assessed as *slight*.

If the dose of adrenaline was increased to 300 μg. the reaction began as above. Later the animals moved restlessly about the cage, eventually lying down in an exhausted manner. Dyspnoea and tachycardia were more marked, and in very severe cases, especially those which proved fatal, a copious bloodstained frothy discharge appeared at the nose. During the stage of exhaustion the animals could be handled without any resentment. The attack usually commenced 10 minutes after the injection ; dyspnoea and tachycardia were marked for 45 to 60 minutes, and prostration lasted from 2 to 4 hours. The animals had recovered in 5 hours. This type of reaction was assessed as *severe*, and the animals appeared to be in the state of shock.

In Group 1, 7·6 mg. of adrenaline were administered in 110 days and 9 animals died while tolerance to 240 μg. (4 minims) of the drug was being developed. Four of the five remaining rats were injected with 1 mg. (16 minims) adrenaline and a very severe reaction was produced. One uninjected rat was killed at the beginning of the test and the remainder at intervals of ½, 1½, 3 and 5 hours after the injections. In a preliminary investigation of a similar nature the animals received no food from 5 o'clock on the night before the experiment and, when the liver was examined the following morning, marked depletion of glycogen was found. Accordingly, all animals in this and subsequent groups received an adequate diet of bran and oats supplemented by bread and cabbage up to the morning of the test, when all food was withdrawn and the animals were supplied with water only. With this regime satisfactory storage of liver glycogen was attained at the beginning of the experiments.

Group 2 consisted of 14 animals which were treated for 100 days with gradually increasing doses until they were adapted to 240 μg. (4 minims) 1:1000 adrenaline hydrochloride. During that period 6·4 mg. of adrenaline were administered and 8 animals survived. One animal served as a control and the remainder received 240 μg. (4 minims) subcutaneously on the day of the test, when they were killed off at hourly intervals for a period of 6 hours. A *slight* reaction only was produced.

Group 3 : Eight animals constituted this series and they had received no previous injections. During the test 240 μg. (4 minims) 1:1000 adrenaline hydrochloride were injected and the animals killed at intervals of ½, 1½, 2, 3, 4 and 5 hours. One uninjected animal was kept as a control. The reactions produced were *slight* in character, even though no previous injections had been given.

Group 4 : Bilateral adrenalectomy was successful in 9 animals in this group. Both adrenals were removed by a dorsal approach under ether anaesthesia, and 10 ml. normal saline injected intraperitoneally immediately following the operation. The animals were placed on the above-mentioned diet containing abundant cabbage for 5 days and 5 ml. normal saline administered intraperi-

toneally every second day. In this way it was hoped that the store of liver glycogen would be built up, while 5 days would not be long enough to allow ectopic adrenal cortical tissue to develop. On the day of the test 240 μg. (4 minims) 1:1000 adrenaline hydrochloride were injected and the rats killed subsequently at intervals of ½, 1, 2, 3 and 5 hours. One animal was not injected and acted as control. The reactions produced were *slight* in all cases.

All animals were stunned by a blow on the back of the head and their throats cut. Blood was collected in heparin tubes for glucose estimations, post-mortem examinations carried out and the adrenals and liver removed for histology.

Adrenals.—All adrenals were treated with osmic acid solution and the sections cut serially at 5–6 μ. Material from a few adrenals was also fixed in a solution of formol-calcium, embedded in gelatine and cut at 11 μ. Some sections were examined for birefringent substances and others were stained for lipoids, using Sudan IV and Sudan black. The Schultz reaction for cholesterol (Carleton and Leach, 1947) was done in a modified manner. Sections 11 μ. embedded in gelatine were treated for 3 days in a solution of 2·5 per cent iron alum and transferred directly to a slide. A solution consisting of equal quantities of glacial acetic acid and concentrated sulphuric acid was kept for 3 days before use and a drop of this mixture added to a coverslip, which was then placed on the gelatine section. Good results were obtained by this method; no air bubbles were formed and charring of the section delayed.

Liver glycogen.—A histological technique was used and the glycogen in the liver lobule of a control animal taken as standard; the disappearance and subsequent regeneration of glycogen in the lobule could be studied at different stages of the reaction. Three histological methods (Best's carmine; Mullen, 1944; Mitchell and Wislocki, 1944) were used and all gave satisfactory results when the individual technical difficulties were overcome. The method described by Mitchell and Wislocki was the one chosen, mainly because of its good photographic properties. It has its own peculiarities, like most glycogen methods, and slight modifications had to be adopted; e.g. the liver when removed from the animal was cut in thin slices and placed in the refrigerator for 15 minutes before fixation. After 4 hours' fixation the slices were removed, trimmed and re-fixed for a further 8 to 14 hours. They were treated subsequently as described by the authors.

Blood sugar.—Blood sugar was estimated by the method of Hagedorn and Jensen.

RESULTS.

Group 1.—*Severe Reaction Stimulated by* 16 *Minims Adrenaline in Partially Adapted Animals.*

Adrenals.—The adrenal gland of the adapted uninjected animal showed a well-marked zone of osmophil-positive lipoid material in the zona fasciculata. Only a few lipoid granules were seen in the cells of the zona glomerulosa, but the usual narrow rim of lipoid material was present in the juxta-medullary layer of the zona reticularis. In the first animal killed ½ hour after a severe adrenaline reaction, vacuoles appeared in the cells of the zona fasciculata and pale lipoid material was seen in the medullary sinusoids and the adrenal vein. As the severity of the adrenaline reaction increased, the remaining animals were clinically

in the state of exhaustion (1½ hours) and the cells of the zona fasciculata were depleted of lipoid. Nevertheless, in this animal the lipoid content of the zona glomerulosa had increased and a prominent subglomerulosal zone was present (Fig. 5). The rats showed signs of recovery in 3 hours and the animal killed at this time had lipoids in the zona fasciculata, which was vacuolated once more. This zone had now united with the zona glomerulosa and the subglomerulosal or sudanophobe zone had disappeared. Five hours after the injection, the last animal had recovered and on examination the glomerulosa and fasciculata reappeared as distinct zones. Lipoid material was present in both, while vacuolation of the cytoplasm of the cells of fasciculata was still prominent. At all times during the reaction lipoid material was detectable in the medullary sinusoids, but it appeared rather yellow-brown in colour and was never so intensely osmophil-positive as in Fig. 3.

Liver.—Glycogen was present in the cells of all parts of the liver lobule of the uninjected adapted animal. Half-an-hour after 16 minims adrenaline the cells around the central vein were completely devoid of glycogen and the nuclei of those cells could not be distinguished at all (Fig. 6). As the reaction became more severe (1½ hours), the extent of the glycogen deprivation was more extensive and the cells around the portal tract and central vein were depleted of their supply. Glycogen was present still in the mid-zone region though the liver at this stage was extremely depleted. During the onset of recovery (3 hours), the mid-zone supply was slightly increased but the picture did not vary much from the previous one. There was almost complete regeneration after 5 hours, only a few cells in the immediate vicinity of the central vein and portal tract lacked glycogen.

Blood sugar.—During the first 1½ hours after the injection there was a rapid rise in blood sugar to the highest level of 340 mg. per cent. This was followed by a gradual decrease to 234 mg. per cent in 3 hours after which the fall was much slower. The blood-sugar level (181 mg.) per cent was still well above the normal value (133 mg.) per cent after 5 hours when the last animal had recovered. This sustained mobilisation of blood sugar is shown in Fig. 1.

Group 2.—Slight Reaction Stimulated by 4 Minims Adrenaline in Adapted Animals.

Adrenals.—The histological appearance of the adrenal in the uninjected animal was similar to the corresponding one seen in Group 1. One hour after the injection clear vacuoles appeared in the cytoplasm af many cells of zona fasciculata and osmophil lipoids were present in the remainder. This picture persisted in all glands and was present 6 hours after the injection. Only a few lipoid granules were seen in the zona glomerulosa of most of this series, apart from that seen in the rat killed at the second hour when a more prominent lipoid zone was observed. However, the lipoid content of this zone was never so marked as that seen in Group 3 animals.

Liver.—Normal glycogen deposits were present in the control and first hour specimens ; depletion began to occur around the central veins in 2 hours and became more marked 1 hour later. The glycogen remained mainly in the mid-zone region at this stage, but it had returned to normal 4 hours after the injection.

Group 3.—Slight Reaction Stimulated by 4 Minims Adrenaline in Normal Unadapted Uninjected Animals.

Adrenals.—The adrenals from the uninjected animal showed the recognized normal histological picture. There was a prominent lipoid zona glomerulosa which was separated from an equally prominent lipoid zona fasciculata by a clear sub-glomerulosal zone. A rather patchy distribution of lipoid was seen in the zona fasciculata 1 hour after the injection, but this was obvious only on high power examination. Many cells were full of lipoids and their cytoplasm uniformly black in colour ; in others lipoid was absent and the cytoplasm appeared grey in colour. Vacuoles containing discrete round black lipoid granules were noticeable in a few cells. Abundant lipoid material occupied the zona glomerulosa and the sub-glomerulosal zone was prominent (Fig. 7). One hour later more cells of the zona fasciculata were depleted of lipoid ; many showed lipoid granules in their cytoplasmic vacuoles, and a prominent lipoid zona glomerulosa was present. A somewhat similar picture was seen 3 hours after the injection, but the vacuolated appearances of the glands at 4 and 5 hours resembled those in Group 2 in that vacuolation of the cells of zona fasciculata was now a prominent feature. Most of the vacuoles contained black lipoid granules though others appeared empty. The cytoplasm of many cells, however, was full of lipoid and lipoid material was present always in the zona glomerulosa (Fig. 8).

EXPLANATION OF PLATES.

FIG. 3.—Rat adrenal. Osmophil-positive lipoid material is shown in the widely dilated medullary sinusoids. The rat died 5 hours after a severe adrenaline reaction. × 75. Osmic acid.

FIG. 4.—Rat adrenal. The juxta-medullary portion of zona reticularis is seen above and the medulla below. Osmophil-positive lipoid, shown in Fig. 1, is present in the medullary sinusoids and can be seen passing downwards from the cortical capillaries. × 250. Osmic acid.

FIG 5 (Group 1).—Rat adrenal 1½ hours after a severe adrenaline reaction. A well-marked lipoid zone is present in zona glomerulosa, but there is depletion of lipoid from zona fasciculata. × 75. Osmic acid.

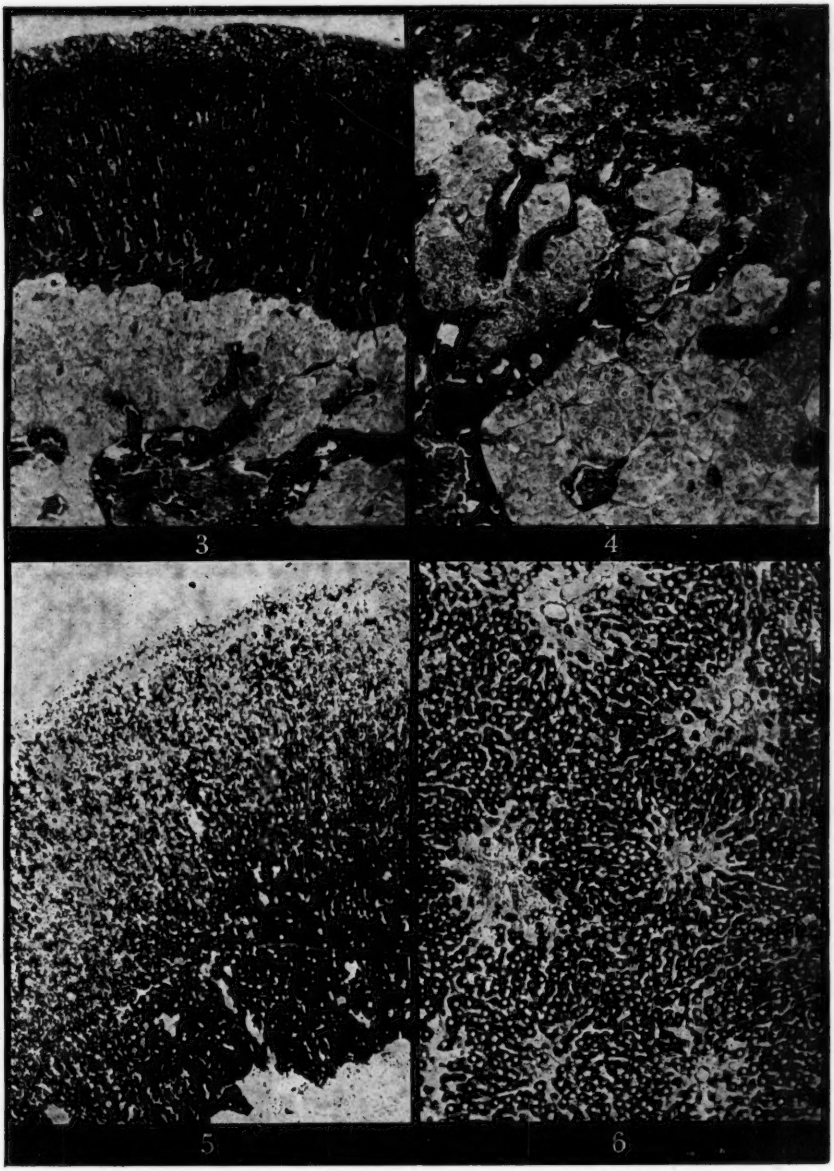
FIG 6 (Group 1).—Liver showing the disappearance of glycogen from the centre of the liver lobule ½ hour after a severe adrenaline reaction. The glycogen is stained black and the nucleus of the cells appears as unstained rings. × 100. Silver impregnation (Mitchell and Wislocki).

FIG. 7.—Rat adrenal one hour after a slight adrenaline reaction (Group 3). The gland appears normal. Osmophil lipoid is shown in both zona glomerulosa and zona fasciculata, which are separated by a clear sub-glomerulosal or sudanophobe layer. High-power examination, however, shows a rather patchy distribution of lipoid in zona fasciculata (see Fig 8), not evident on low magnification. × 20. Osmic acid.

FIG. 8.—Rat adrenal 4 hours after a slight adrenaline reaction (Group 3). The zona glomerulosa is seen to the right and the larger zona fasciculata to the left. Dense black osmophil lipoids are present in many cells, the unstained nuclei of which are clearly seen. Most cells are vacuolated, some of the vacuoles contain discrete black lipoid granules, others are completely empty and their nuclei pushed to the periphery of the cell. × 250. Osmic acid.

FIG. 9.—Rat adrenal 6 hours after a severe reaction to 10 minims 1 : 1000 adrenaline hydrochloride. The animal was in the exhaustion phase of the adaptation syndrome (Selye, 1946). Osmophil lipoids are present only in the zona glomerulosa. The zona fasciculata has a greyish coloration and is lipoid-deficient (see Fig. 10). × 20. Osmic acid.

FIG. 10.—High-power of Fig. 9. The zona glomerulosa is seen to the right and zona fasciculata to the left. Very few cells in the zona fasciculata contain osmophil lipoids, most of them have a greyish appearance. Black lipoid granules but no vacuoles are seen in some cells. The nuclei of the cells appear as white unstained circles. × 250. Osmic acid.



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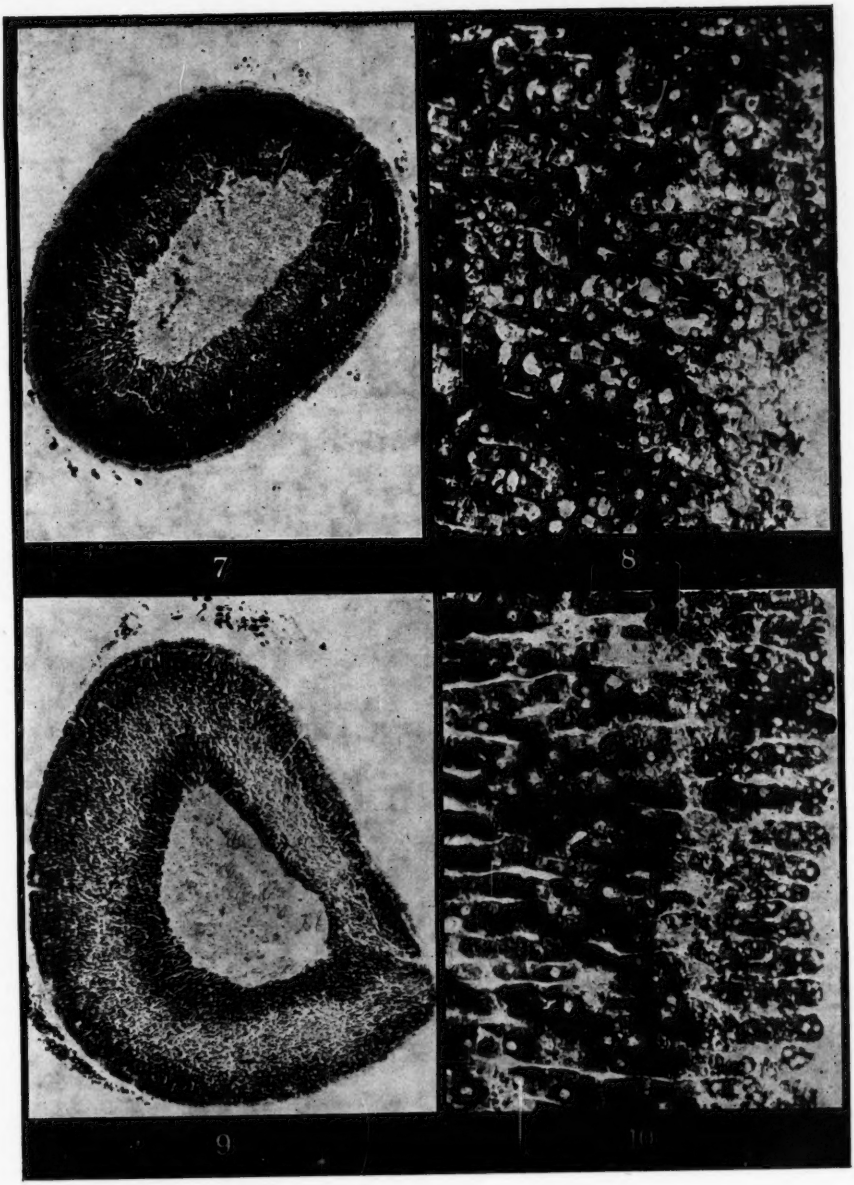

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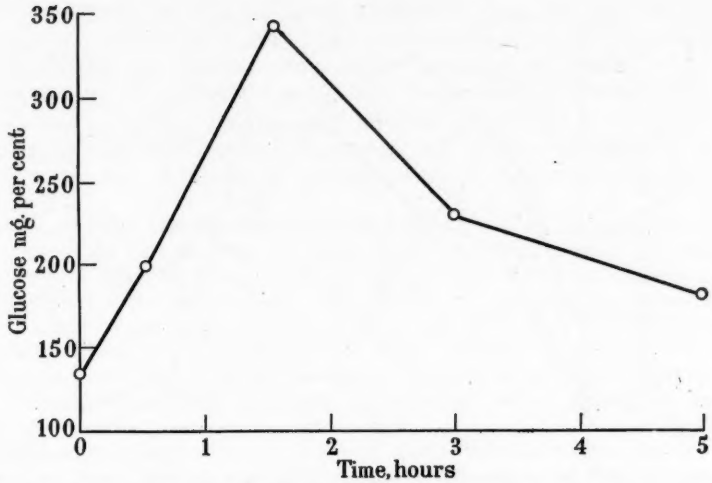

FIG. 1 (Group 1).—Blood sugar changes in the rat following a severe adrenaline reaction.

Liver.—When the liver was examined ½ hour after the injection, glycogen depletion had occurred around the central vein. The depletion became more marked in 1½ hours when glycogen was limited to the mid-zone region, but deposition of glycogen occurred 1 hour later and was complete 4 hours after the injection.

Group 4.—Slight Reaction Stimulated by 4 Minims Adrenaline in Untreated Adrenalectomized Animals.

Adrenals.—There was no evidence of ectopic cortical tissue when the animals were examined at post-mortem.

Liver.—The normal glycogen content of the control animal began to disappear from the cells around the central vein ½ hour after the injection. Depletion was more marked after 1 and 1½ hours, when glycogen was present only in the mid-zone region. This appearance remained in subsequent sections at 2, 3, 4 and 5 hours, when no evidence of glycogen regeneration was noticeable.

Blood Sugar Changes in Groups 2, 3 and 4.

Fig. 2 shows the blood sugar curves of the three groups after slight reactions. The curve of the unadapted animals (Group 3) shows once more the continued mobilization of the blood sugar even 5 hours after the injection. As might be expected, there is a much slower rise of the curve in the adapted group (Group 2) but mobilization of blood sugar is still present. The curve of the adrenalectomised group (Group 4) shows a rapid rise with a maximum level of 250 mg. per cent in one hour, after which the blood sugar falls rapidly to normal in 3 hours. Thus the failure to mobilize blood sugar is the characteristic and outstanding feature of this curve.

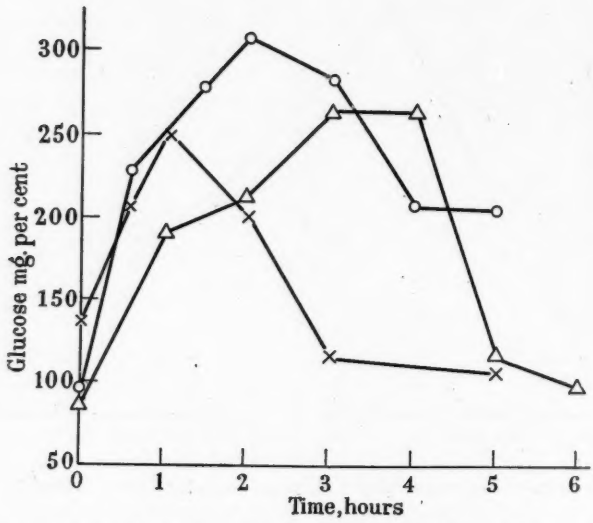

FIG. 2.—Blood sugar changes in Groups 2, 3 and 4 following a slight reaction to 4 minims of 1 : 1000 adrenaline-hydrochloride.

DISCUSSION

Great advances have been made within recent years in identifying a large number of chemical substances associated with the adrenal cortex. Many of them are related to the male and female sex hormones while others have assumed importance because of the part they play in the regulation of sugar and salt metabolism. The latter group, referred to as "corticoids," are derived from the basic substance corticosterone. The substances 11-dehydro-corticosterone and 11-dehydro-17-hydroxy-corticosterone (Kendall Compound E or Cortisone) are associated with sugar metabolism and are called the sugar-active corticoids, while 11-desoxy-corticosterone and 11-desoxy-17-hydroxy-corticosterone are associated with electrolyte balance and are referred to as salt-active corticoids.

Various biological reactions have been devised to test the activity of corticoids (Dorfman, 1949) and a few of them are shown below :

(1) "Ingle" work test (Ingle, 1936 ; 1944) for sugar-active corticoid is a measure of the total work yielded by the muscle of an adrenalectomized rat when treated with this substance.

(2) The life preservation test is a measure of the ability of salt-active corticoid to preserve the life of adrenalectomized mice or rats.

(3) The insulin insensitivity reaction depends on the ability of sugar-active corticoid to prevent the hypoglycaemic action of insulin when administered to normal or adrenalectomised starving animals (Selye and Dosne, 1939 ; Grattan and Jensen, 1940 ; Grattan, Jensen and Ingle, 1941).

(4) This test depends on the ability of sugar-active corticoid to raise the level of liver glycogen in adrenalectomized or normal starving animals (Reinecke and Kendall, 1942, 1943; Bergman and Klein, 1943; Long, Katzin and Fry, 1940; Long, 1942; Tepperman, Engel and Long, 1943).

Since liver glycolysis, with depletion of liver glycogen, results from injections of adrenaline, the bio-assay test involving the regeneration of liver glycogen was the method of choice in the present investigation and indeed has proved satisfactory.

The experiment was so arranged that Groups 2, 3 and 4 served to identify the nature of the lipoids liberated from the adrenal cortex after adrenaline and also as control series for Group 1 and for one another. Animals in Groups 1 and 2 were adapted in the same manner until the day of the test when those in Group 2 received their accustomed dose of 4 minims and the Group 1 rats had 16 minims adrenaline. Thus, the adrenal changes which usually occurred in the animals of both Groups 1 and 2 during the process of adaptation are represented by Group 2 rats; hence those seen in Group 1, following severe adrenaline reactions (16 minims), can be more accurately assessed. Adrenals from the animals killed at the beginning of each experiment served to control the changes which followed injections in each group. Group 3 consisted of uninjected normal rats. The adrenals from the control animals of Groups 1 and 2 varied slightly from those of Group 3 in that very little lipoid was present in zona glomerulosa, but in all other respects the adrenals from the uninjected animals of Groups 1, 2 and 3 produced similar histological pictures following osmic acid treatment. Group 4 acted as a control for the regeneration of liver glycogen which occurred in Groups 1, 2 and 3 after the glycolytic action of adrenaline. In all groups the animals received and took food up to the commencement of the injection, when food was withdrawn. This ensured satisfactory storage of liver glycogen at the beginning of the experiment, and the failure to regenerate liver glycogen in the adrenalectomized Group 4 animals which had taken food up to the time of the experiment proved that the deposition of liver glycogen was dependent upon intact adrenals.

Correlation of the Histological Changes in the Adrenals and Liver with the Blood-Sugar Curve following Slight and Severe Adrenaline Reactions.

Minimal histological changes were observed in the adrenals of Group 2 and 3 animals following a *slight* adrenaline reaction. Vacuoles occurred in the cytoplasm of the cells of zona fasciculata and osmophil lipoids appeared in the medullary sinusoids and adrenal vein. These adrenal changes were associated with the disappearance of liver glycogen which later regenerated 4 hours after the injection. There was mobilization of the blood sugar which in Group 3 was still elevated 5 hours later.

More extensive changes were observed in the adrenals, liver glycogen and blood sugar of Group 1 animals following a severe adrenaline reaction. Lipoid material began to disappear from the zona fasciculata within ½ hour of the stimulating injection and vacuoles appeared in the cells. There was dilation of the blood vessels in the zona reticularis and liberation of lipoid material into the medullary sinusoids and adrenal vein. As the severity of the adrenaline reaction increased and the animals showed evidence of extreme exhaustion (1½ hours),

the zona fasciculata became depleted of lipoid (Fig. 5). At this stage a well-marked osmophil-positive reaction was present in the zona glomerulosa and lipoid material was seen once more in the medulla. The reappearance of lipoid in the zona fasciculata and the fusion of this zone with zona glomerulosa was an interesting feature of the early clinical recovery phase. When the animal had fully recovered clinically in 5 hours, the separation of zona glomerulosa and fasciculata as two distinct zones was seen once more, though it should be noted that the adrenal had not returned to normal. There was depletion of cholesterol (negative Schultz reaction) from the zona fasciculata, but the zona glomerulosa still gave a well-marked positive Schultz reaction. During the whole period of the experiment, lipoid material was detectable continuously in the adrenal medulla. When the liver sections stained for glycogen were examined at corresponding times, glycolysis followed the adrenaline injection and resulted in the disappearance of liver glycogen from the central vein and portal tract. This depletion was most marked during the period of extreme exhaustion (1½ hours), but after 3 hours, regeneration of liver glycogen occurred and this was almost complete at 5 hours. The adrenal and liver glycogen changes were associated with mobilization of blood sugar which was still elevated 5 hours after the injection (Fig. 1).

The experiment showed that variations occurred in the rat adrenals in response to *slight* and *severe* adrenaline reactions, and osmophil lipoid material was liberated immediately and continued to be liberated for many hours afterwards. This was associated with mobilization of blood sugar and regeneration of liver glycogen. Since sugar-active corticoids possess those properties it was very probable that this substance formed an important constituent of the lipoid. Conclusive proof of this was afforded by animals after adrenalectomy in which the liver glycogen failed to regenerate and the blood sugar never was sustained, but fell rapidly to normal levels (Fig. 2).

The investigation thus confirms the work of Vogt (1943, 1944, 1945) and Paschkis, Cantarow, Walkling, Pearlman, Rakoff and Boyle (1948) that appreciable quantities of cortical hormone, especially sugar-active corticoid, are present in adrenal vein blood. Unfortunately, in the absence of a suitable bio-assay technique, it has not been possible to decide if salt-active corticoid is present also in the secretions of the adrenal cortex following stress, although it has been suggested that the site of formation of this substance is the zona glomerulosa (Deane and Greep, 1949).

The Significance of the Adrenal Response to Severe and Slight Adrenaline Reactions.

When the adrenal glands from normal resting rats are examined by different histo-chemical methods, phospholipines (Cain and Harrison, 1950), Schultz, Sudan black, Sudan IV, birefringence and osmic acid, the general distribution of lipoid in all zones is similar irrespective of the method used. However, Cain and Harrison demonstrated that the lipoid content of the zona fasciculata varies in different animals, and such variations in the lipoid pattern of this zone are seen even in "standard" normal animals kept isolated and protected from many of the daily non-specific stimuli which are known to affect the adrenal glands. Generally, the zona glomerulosa of normal rats contains a well marked zone

of lipoid material which is separated from the lipoid of the zona fasciculata by a clear sub-glomerulosal or sudanophobe zone. In the uninjected adapted animal of Groups 1 and 2 only a few lipoid granules were seen in the zona glomerulosa and this appearance was noted in most of Group 2 rats, and has been reported by Bennett (1940) in the normal cat adrenal.

In the present investigation the appearance of the adrenal glands stained by osmic acid was found to vary with the severity and duration of the adrenaline reaction and with the time after stimulation when the adrenals were examined. When the reaction was slight (Groups 2 and 3) or when the animal was adapted (Group 2), the adrenals varied little from normal, but vacuolation of the cells of zona fasciculata was prominent. The demand for cortical hormone was relatively small and that it was adequately met by the gland is shown by the ease with which liver glycogen was regenerated and blood sugar mobilized. This type of response is seen also in fasting animals and corresponds to the Type 2 response of Sayers and Sayers (1949), and Sayers (1950), which is described as the response of the adrenal to " a very slow gradual change in the internal or external environment." When the degree of stress was of a severe, sudden, but temporary nature, as seen in animals following a severe adrenaline reaction (Group 1), there was a sudden, temporary demand for sugar-active corticoid which was seen to be forthcoming. It was associated with a variable histological picture when the gland was examined at different stages of the reaction. The adrenal changes in this series correspond with the Type 1 response which can be produced by a single injection of ACTH or " follows an acute non-fatal haemorrhage " (Sayers and Sayers). However, it must be appreciated that the adrenal response to a sudden temporary period of stress (Sayers Type 1 response) will depend on the degree of adaption developed by the animal to the response. In the present investigation Group 1 animals had been adapted to adrenaline to some degree, and hence they were able to deal more efficiently with a sudden temporary period of stress induced by injecting a larger dose of adrenaline. The gland apparently was able to recover and this suggests that " discharge phase " is a more appropriate term than " exhaustion phase " for the adrenal state when lipoid in the zona fasciculata is depleted.

The recovery of the adrenal gland from adrenaline stress can be delayed if the animal loses its adaption to the drug by repeated injections of adrenaline. Such an animal when subjected to a severe adrenaline reaction shows depletion of lipoid material from zona fasciculata and marked accumulation of lipoid in zona glomerulosa. In a recent series this picture was seen 2 hours after a severe adrenaline reaction when the animals had lost their adaptation to adrenaline. The same histological picture was found 7 hours after the injection and no evidence of recovery was evident (Fig. 9 and 10). If such a severe stress was continued death of the animal would result. This type of adrenal response is found after lethal doses of toxins and severe burns, and corresponds to the Type 3 response (Sayers and Sayers).

The adrenal changes shown demonstrate the variable picture which might be obtained in response to severe stress in the form of adrenaline injections and the liberation of sugar-active corticoid which is brought about. This endogenous liberation of corticoid may be the basis of the temporary success of adrenaline and other non-specific agents in the treatment of rheumatoid arthritis and of post-operative control of blood pressure. Little is known of the effect on the

adrenal of multiple daily injections of adrenaline, but preliminary investigations on this subject have resulted in the appearance of haemorrhagic adrenals in some instances.

Osmic Acid as a Histo-chemical Stain for Adrenals.

Osmic acid was used in the present investigation mainly because the lipoid material in the medullary sinusoids was seen only in osmic acid preparations. The presence of lipoid material in the zona fasciculata of the normal animal (secretory phase, Cain and Harrison) and its subsequent depletion (discharge phase, Cain and Harrison) has been shown with osmic acid, and the significance of those changes is enhanced when the combined histological bio-assay technique is used. Since the sections can be cut serially at 5–6μ with optimum zoning effect, more intimate examination of individual cells is possible and intracellular lipoids can be seen (Fig. 8 and 10), while vacuolation of the cell cytoplasm is demonstrated more easily by osmic acid than by Sudan IV or Sudan black preparations. Nevertheless, the non-specificity of osmic acid as a lipoid stain was encountered in the investigation. In some instances it did not produce the clear-cut zoning arrangement of Sudan black or Sudan IV, and the zona fasciculata in the discharge phase occasionally presented a greyish appearance (Fig. 9), although high power examination clearly demonstrates the absence of lipoid material (Fig. 10). In addition, like many other lipoid stains it is not such a sensitive guide to adrenal cortical activity nor as good an indication of return to normal as the Schultz reaction (Robinson and Yoffey, 1950).

CONCLUSIONS.

The adrenal cortex responds to the injections of 1:1000 adrenaline-hydrochloride by an immediate and continued liberation of material containing sugar-active corticoids.

The histological bio-assay technique used to identify this material is described.

The appearance of the adrenal cortex of the rat examined by osmic acid varies with the severity and duration of the adrenaline reaction and the time after stimulation when the adrenals are examined.

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AN ELECTROPHORETIC ESTIMATION OF THE CORRELATION BETWEEN THE CHOLESTEROL CONTENT AND THE MATERIAL EXTRACTABLE FROM β-GLOBULIN IN NORMAL AND DIABETIC SERA.

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THE cholesterol in serum is known on electrophoresis to migrate mainly with the β-globulin fraction. Furthermore, the effect of extracting lipids from serum on the electrophoretic pattern is to reduce the area of the β-peak. This work was undertaken to correlate these phenomena, and to attempt to find any significant differences between normal and diabetic sera.

METHODS.

The sera used in the present work were obtained in connection with research (to be published) on various aspects of diabetes mellitus at this university. All diabetic patients were well controlled and had no (i) cardiac failure or history of cardiac decompensation, (ii) albuminuria or history of nephritis or other renal disease, (iii) icterus or history of jaundice, (iv) alcoholism, (v) cirrhosis of liver or chronic cholecystitis, (vi) myxoedema or thyrotoxicosis. All patients were ambulatory. Normal sera were obtained from healthy donors. All blood was drawn at the same time of day.

Electrophoretic analyses were carried out in a modified form of the apparatus (constructed by Adam Hilger, Ltd.) described by Tiselius (1937) and equipped with the cylindrical lens and diagonal edge type of optical system introduced by Philpot (1938). Photographs were taken with the mercury 546 mμ. source of illumination and with both inclined edge and wire, but the latter was always used for planimetry (Svensson, 1939). Serum was diluted six or seven times with veronal buffer at pH 8·6 and ionic strength 0·1, then dialysed in a cellophane membrane at approximately 0° C. for at least two days against 2 litres of the same buffer, which was later used to fill the electrode vessels. In view of the expense of veronal it was recovered from the used buffer by precipitation with dilute hydrochloric acid followed by crystallization from alcohol. The standard 11 ml. Tiselius cell with double-length centre section was used. Each pattern was enlarged and a tracing made, which was divided up according to Pedersen's (1940) method. Each peak was measured at least four times with a planimeter and the figures averaged. The analysis was calculated as described by Longsworth (1942).

Lipids were removed from sera by a modification of the Hardy-Gardiner method described by Hartley (1925).

The Pregl technique was used for the micro-Kjeldahl nitrogen estimations and a factor of 6·25 to convert nitrogen to protein. Total cholesterol was determined by the method of Sperry and Schoenheimer (1934).

RESULTS.

Table I shows the electrophoretic analyses of the sera expressed as a percentage of the total refractive area after subtracting the extra-gradient contribution.

TABLE I.—*The Effect of Lipid Extraction by the Hardy-Gardiner Method on the Electrophoretic Analyses of Normal and Diabetic Sera.*

Patient number.	Dialysis time (days).	Total cholesterol (mg. %).	Electrophoretic analysis as percentage protein.					β-globulin concentration (g./100 ml).
			Alb.	α_1.	α_2.	β.	γ.	
Diabetic sera before removing lipids.								
2 IM	3	215	55·4	5·8	9·2	11·7	17·9	0·95
3 EB	2	262	54·2	7·0	10·5	16·3	12·0	1·12
4 CR	2	177	51·2	7·0	10·4	15·1	16·3	1·12
8 AT	3	138	51·4	7·9	9·5	14·5	16·7	1·04
9 DR	3	413	46·7	6·7	11·3	16·2	19·1	1·27
12 DS	2	253	44·4	6·6	11·5	20·5	17·0	2·15
13 BB	2	224	51·5	9·4	11·8	15·7	11·6	1·18
14 JS	3	215	48·2	8·0	11·7	17·2	14·9	1·27
15 MS	4	—	51·1	8·5	12·6	16·0	11·8	1·47
Normal sera before removing lipids.								
17/8	2	212	56·8	5·0	7·4	15·4	15·4	1·20
21/2	2	203	57·9	4·7	8·3	13·9	15·2	1·06
23 RD	2	207	55·8	6·9	10·3	13·9	13·1	1·17
24 MF	3	188	61·7	3·8	6·3	12·8	15·4	0·99
Diabetic sera after removing lipids.								
2 IM	2	—	58·9	6·3	10·0	6·8	18·0	—
3 EB	5	—	56·0	7·7	13·0	8·1	15·2	—
4 CR	4	—	56·2	6·3	11·7	7·9	17·9	—
8 AT	5	—	51·2	7·8	12·3	9·1	19·6	—
9 DR	4	—	49·1	5·8	16·0	8·0	21·1	—
12 DS	2	—	46·5	8·0	16·0	11·2	18·3	—
13 BB	5	—	56·0	8·5	14·9	8·1	12·5	—
14 JS	5	—	50·0	8·9	14·6	10·6	15·9	—
15 MS	3	—	56·0	5·9	17·1	9·4	11·6	—
Normal sera after removing lipids.								
17/8	4	—	57·4	5·2	11·3	10·0	16·1	—
21/2	2	—	58·5	5·5	11·5	7·0	16·5	—
23 RD	2	—	59·4	5·9	11·7	9·4	13·6	—
24 MF	2	—	62·4	5·0	11·0	7·1	14·5	—

In order to obtain a clearer impression of the effect of lipid removal on the individual globulins use is made of the fact that albumin is virtually lipid-free. Thus, the ratio—

$$\frac{\text{Albumin/Globulin in unextracted serum}}{\text{Albumin/Globulin in lipid-extracted serum}}$$

measures the fractional reduction in area of the globulin peaks on the electrophoretic schlieren pattern as a result of lipid removal. Table II gives these values.

TABLE II.—*The Fractional Reduction in Concentration (Area) of Serum Globulins as a Result of Lipid Removal.*

Patient number.	Globulins. α_1.	α_2.	β.	γ.	Total globulins.
		Diabetic sera.			
2 IM	1·02	1·02	0·55	0·95	0·87
3 EB	1·07	1·20	0·48	1·23	0·93
4 CR	0·82	1·02	0·48	1·00	0·82
8 AT	0·99	1·30	0·63	1·18	1·01
9 DR	0·82	1·35	0·47	1·05	0·91
12 DS	1·16	1·33	0·52	1·03	0·92
13 BB	0·83	1·16	0·48	0·99	0·83
14 JS	1·07	1·20	0·59	1·03	0·93
15 MS	0·64	1·24	0·54	0·90	0·82
		Normal sera.			
17/8	1·03	1·51	0·64	1·03	0·98
21/2	1·16	1·38	0·50	1·08	0·98
23 RD	0·80	1·07	0·63	0·97	0·86
24 MF	1·30	1·73	0·55	0·93	0·97
		Means of all above figures.			
	0·98	1·27	0·54	1·03	0·91

The quantity—

$$\left\{1 - \frac{\text{A/G unextracted serum}}{\text{A/G lipid-free serum}}\right\} \text{ β-globulin, concn. g./100 ml.}$$

was taken as an approximate measure of the material removed from β-globulin by the lipid-extraction technique used in this work. A correlation coefficient was calculated between this quantity and the total cholesterol figures for the sera as described by Tippett (1931). The twelve available results gave a positive correlation of 0·47, corresponding to a level of significance of 0·1. No distinction was made between the diabetic and normal sera when determining this correlation for reasons dealt with in the discussion.

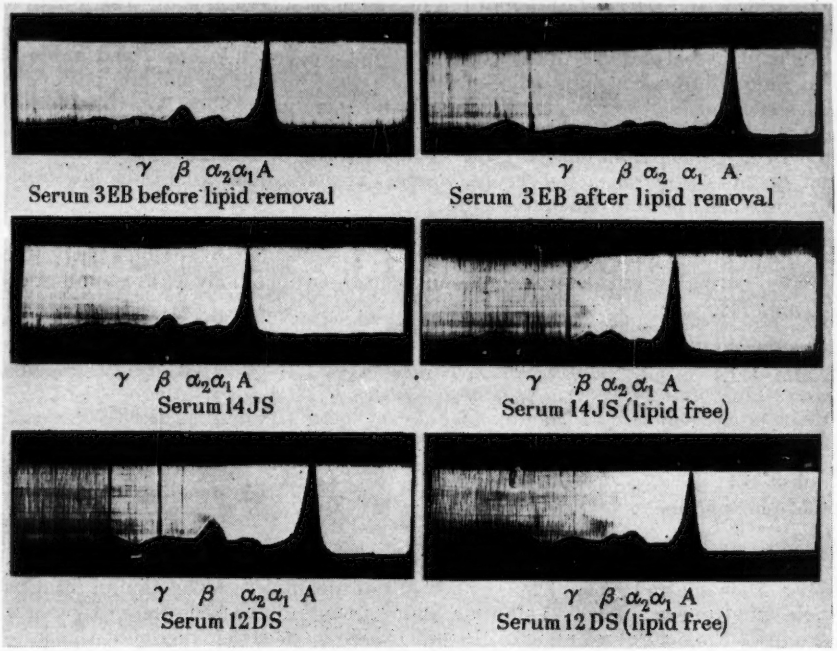

FIG. 1 shows typical electrophoretic patterns illustrating the effect of lipid removal from serum by the Hardy-Gardiner method. (Ascending patterns).

DISCUSSION.

In this work the comparison of protein levels in normal and diabetic sera does not concern us. The analyses given here are a part of a wider survey of diabetes mellitus taking place in this university. However, well-controlled diabetic serum is not grossly abnormal. Albumin is generally lowered while globulins are raised, the total protein level remaining normal. In support of this, Lewis, Schneider and McCullagh (1944) found, in their electrophoretic analyses of diabetic plasma, that, after treatment, all protein levels returned to normal except when complicated by diabetic retinitis, when electrophoretic patterns became "nearly normal" on a high protein diet. In general, well controlled diabetic serum tends to contain slightly more cholesterol and β-globulin than normal, but in view of the experimental errors involved in the present work it was decided to combine diabetic and normal figures in working out a correlation coefficient between total cholesterol and material extractable from β-globulin. There is no justification for assuming any significant difference between the diabetic and normal sera in the effect of lipid extraction on the electrophoretic patterns.

The Hardy-Gardiner method of extracting serum lipids was chosen for its thoroughness combined with gentleness; it removes all cholesterol, whether free

or esterified, and a large fraction of the phospholipids (Delsal *et al.*, 1950), yet, as shown by Tayeau *et al.* (1949), it does not harm antibodies.

The main features of a lipid-extracted serum are shown in Fig. 1 and Table II. The β-globulin peak is markedly reduced in area, while the α_2-globulin peak actually shows an increase in area. The α_1- and γ-globulins show, on the average, no marked change in area. Furthermore, if electrophoresis is carried on about 50 per cent longer than normally the α_2-globulin can be resolved into at least two components in the extracted serum, whereas under similar conditions it remains unresolved in the unextracted serum.

These observations must be brought into line with previous knowledge of the lipid content of serum fractions. Blix, Tiselius and Svensson (1941) fractionated normal serum electrophoretically and their lipid analyses are summarized in Table III.

TABLE III.—*Lipids in Electrophoretically Separated Normal Serum Fractions (taken from Blix, Tiselius and Svensson,* 1941).

	Albumin. g./100 g.	α-globulin. g./100 g.	β-globulin. g./100 g.	γ-globulin. g./100 g.
Cholesterol	1·07	4·45	8·67	0·41
Lipid-phosphorus . . .	0·09	0·29	0·40	0·04

However, this analysis is now considered to be inaccurate (e.g. McFarlane, 1949 ; Marrack and Hoch, 1949) as a result of incomplete separation of fractions or electro-osmosis. Purified albumin and γ-globulin have been found to contain very little cholesterol (Cohn *et al*, 1946). There is, however, no doubt that most of the lipids occur in the β-globulin and a smaller amount in the α-globulin. (Note : The α-globulin of Blix, Tiselius and Svensson (1941) must be identified with the α_2-globulin fraction of the present work and their albumin contained the α_1-globulin.)

The fact that the α_1- and γ-globulins are not extracted, as far as can be judged from the present data, fits in with our knowledge of the lipid-free character of γ-globulin, but less is known of the composition of α_1-globulin since it is difficult to purify. However, the present technique fails to detect extractable material in α_1-globulin.

The marked decrease in area of the β-globulin peak as a result of removal of lipids is not surprising because this globulin contains most of the lipids in serum, but the decrease seems to be out of proportion to the actual lipid content, as far as can be judged without knowing the specific refraction increments involved. It is known that β-globulin is a mixture of lipoproteins and lipid-free proteins, and it is possible that the decrease in area of the β-peak is due to loss of lipoproteins and not merely due to loss of lipids. The small β-peak left after the Hardy-Gardiner extraction is probably made up of the original lipid-free proteins.

The fate of the proteins left after extracting the lipids from β-globulin lipoproteins is probably connected with the increase in the α_2-globulin peak. Since α_2-globulin contains some lipids, a decrease in area of the corresponding peak on the electrophoretic pattern would be expected, but not to such a marked extent as the β-globulin with its higher lipid content. This expected decrease probably occurs, but is more than compensated for by the inclusion of the proteins derived

from the β-globulin lipoproteins. The net result is thus an increase in size of the α_2-peak. The increase in size of the α_2-peak could be due to denatured serum proteins, but a more probable explanation is that removal of lipids from lipoproteins produces a smaller molecule, and the splitting of cholesterol ester linkages leaves the protein molecules split off more negatively charged than before. The combination of smaller size and increased negative charge confers increased negative mobility, and the proteins involved now migrate with a velocity comparable to α_2-globulin. Mobilities were not determined in connection with this work, but such measurements at a range of pH values would be of great interest. In this connection Wuhrmann *et al.* (1950) find that " if the β_2-globulin shows a marked decrease in electrophoretic mobility it is a sign of changed protein structure as well as a consequence of the high lipid content," whereas in the present case we have the opposite extreme of lipid removal causing greatly increased mobility. The effect of lipid-phosphorous has been ignored here because its concentration is much less than that of the cholesterol.

The α_2-globulin is no longer electrophoretically unresolved after lipid extraction, but clearly exhibits at least two sub-fractions if electrophoresis is carried on about 50 per cent longer than usual. This heterogeneity is probably due to the inclusion of proteins derived from β-globulin lipoproteins as described above, and also to the fact that lipid-extracted α_2-globulins will now have a higher mobility than their parent lipoproteins.

The correlation coefficient deduced between cholesterol and material extractable from β-globulin corresponds to an approximate level of significance of 0·1. This correlation is probably in reasonable agreement with our knowledge of the cholesterol content of β-globulin. A higher level of significance would probably be found if allowance were made for the phospho-lipid contribution and the variable fraction of the serum lipids found in the α_2-globulin. Specific refraction increments have been assumed to be the same for all the species involved, and the reduction in area of the β-globulin peak appears to be due to lipoprotein rather than lipids alone. All these factors can obscure the true correlation.

SUMMARY.

Diabetic and normal sera have been electrophoretically analysed. A correlation coefficient has been deduced between total cholesterol levels and material extractable from the serum by the Hardy-Gardiner method, expressed by the reduction in area of the β-globulin peak on the electrophoretic schlieren pattern. No significant difference was found between the normal serum and that of the well-controlled diabetic patients in that respect. The level of significance of the correlation is given and discussed.

The effect of lipid extraction on the sera as indicated by the electrophoretic analyses was the marked reduction in area of the β-peak, an increase in area of the α_2-globulin peak, and no detectable effect on the α_1- and γ-globulin peaks. These observations are discussed in terms of our knowledge of the lipid content of serum fractions. In particular, the increase in area of the α_2-globulin peak is considered to be due to inclusion of proteins originally present in the β-globulin of unextracted serum as lipoproteins. The reduction in area of the β-globulin peak is ascribed to removal of lipoproteins, and not merely to removal of lipids. On prolonged electrophoresis the α_2-globulin peak of a lipid-extracted serum can

be resolved into components not observable under similar conditions in the original unextracted serum.

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THE EFFECT OF CORTISONE ON WOUND HEALING.

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THE review of Arey (1936) covers the many and various factors influencing wound healing. In particular, he quotes the reported effects of adrenal extracts and of suprarenal gland stimulation as studied by several authors, and emphasizes the disagreement in their results. Voronoff and Bostwick (1918) and Aievoli (1923) agree that direct application of suprarenal gland extracts to wounds results in the formation of rich granulation tissue. Kosdoba (1934), on the other hand, found that any method that increased the amount of circulating adrenal hormones, e.g. homotransplants or stimulation of the glands, resulted in a prolongation of the healing period.

More recently, Ragan, Grokoest and Boots (1949) drew attention to the well-known fact that in patients exhibiting hyperadrenalism, wounds healed poorly. They also noticed that in those patients receiving ACTH, open wounds, incised abscesses and biopsy sites failed to show normal granulation tissue and were slow to heal. Ragan, Howes, Plotz, Meyer and Blunt (1949) in animals and Creditor, Bevans, Mundy and Ragan (1950) in man showed that the administration of ACTH depressed the formation of granulation tissue in experimentally produced wounds. On the assumption that ACTH stimulates the adrenals to produce cortisone or cortisone-like substances, Blunt, Plotz, Lattes, Howes, Meyer and Ragan (1950) and Ragan, Grokoest and Boots (1949) studied the effect of cortisone on experimentally produced wounds and fractures in animals. These authors found that the reparative processes were greatly depressed, and concluded that the response represented an " inhibition of reactivity."

Acute inflammatory changes are so essentially a part of the process of wound healing that it is of interest at this stage to review the effects of cortisone on these changes. Selye (1949), by injecting formalin subcutaneously into rats, showed that both the acute and self-maintaining chronic inflammatory responses were depressed by cortisone. Jones and Meyer (1950) showed that the inflammatory response to chemical burning of the rabbit's cornea was subdued when cortisone was injected locally into the conjunctiva. Allergic skin responses characterized by hyperaemia and local oedema have also been shown to be suppressed by prior administration of cortisone and ACTH (Long and Miles, 1950; Derbes, Dent, Weaver and Vaughan, 1950; Harris and Harris, 1950; Humphrey, unpublished).

The purpose of the present investigation was to find how cortisone suppressed the process of healing in experimental skin wounds. In Part I the effects of cortisone on wound healing in different animals is described, and in Part II some observations on the effects of cortisone on intracutaneous histamine and a solution of leucotactic peptides are described.

PART I.

A. *The Effect of Cortisone on the Healing of Skin Wounds in Rats.*

Male white rats weighing between 150–250 g. were divided into two groups of 14 each. Group I received 2·5 mg. (approx. 12·5 mg./kg. body weight) cortisone acetate (Merck) daily by intramuscular injection ; the controls, Group II, received injections of saline. The rats from both groups were anaesthetized 24 hours after the injections started and the wounds made. The skin was first shaved and then cleaned with soap, water and spirit. A fold of skin on the back was then pinched up and a leather punch crushed through the two thicknesses of skin. The result was two equally-sized wounds, 5·0 mm. in diameter, involving the skin, panniculus adiposus and panniculus muscularis. The rats were then returned to their cages without any dressing. Both groups of rats were fed liberally on Parkes No. 41 diet.

On days 1, 3, 5, 7, 9, 11 and 13 two animals from each group were killed and the wounds examined histologically. The material obtained was fixed in both 10 per cent formol-saline and 85 per cent alcohol. The formol-fixed material was stained with Ehrlich's acid haematoxylin-eosin, Weigert's elastica, van Gieson, Sylven's method for metachromasia, and by Gomori's method for reticulin fibres. The alcohol-fixed material was processed for alkaline phosphatase by a combination of the methods of Gomori (1939) and of Kabat and Furth (1941).

Results.—Ideally, in this sort of experiment, the results should be assessed on statistical data. This was not possible owing to a shortage of cortisone, but the results of this experiment made it clear that cortisone, in the doses used, had no dramatic effects either on the healing time or in the quality of the granulation tissue formed in the rat. There was a suggestion, however, that in some of the cortisone-treated animals the amount of granulation tissue was reduced (Fig. 1*a* and *b*).

The cortisone-treated rats also showed marked thymic involution, enlargement of the liver due to increased glycogen storage, and changes in the quality of the skin. The latter were noticeable in the living animal, the skin becoming looser and less elastic. Histologically the changes in the skin consisted of a

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EXPLANATION OF PLATES.

FIG. 1*a*.—Rat. Control. A 9-day-old wound showing maturing granulation tissue. Compare the thickness of this wound with that in Fig. 1*b*. H. and E. × 45.

FIG. 1*b*.—Rat. Cortisone-treated. A 9-day-old wound. The granulation tissue present is normal but less abundant. H. and E. ×45.

FIG. 2*a*.—Rat. Control. The panniculus adiposus is prominent, and the hair follicles are seen to extend almost down to the fatty layer. H. and E. × 45.

FIG. 2*b*.—Rat. Cortisone-treated. The appearance of the skin after 12 days' treatment. The panniculus adiposus is absent and the hair follicles do not penetrate very deeply. H. and E. × 45.

FIG. 3*a*.—Rabbit. Control. The appearance of a large full thickness skin wound on the 16th day.

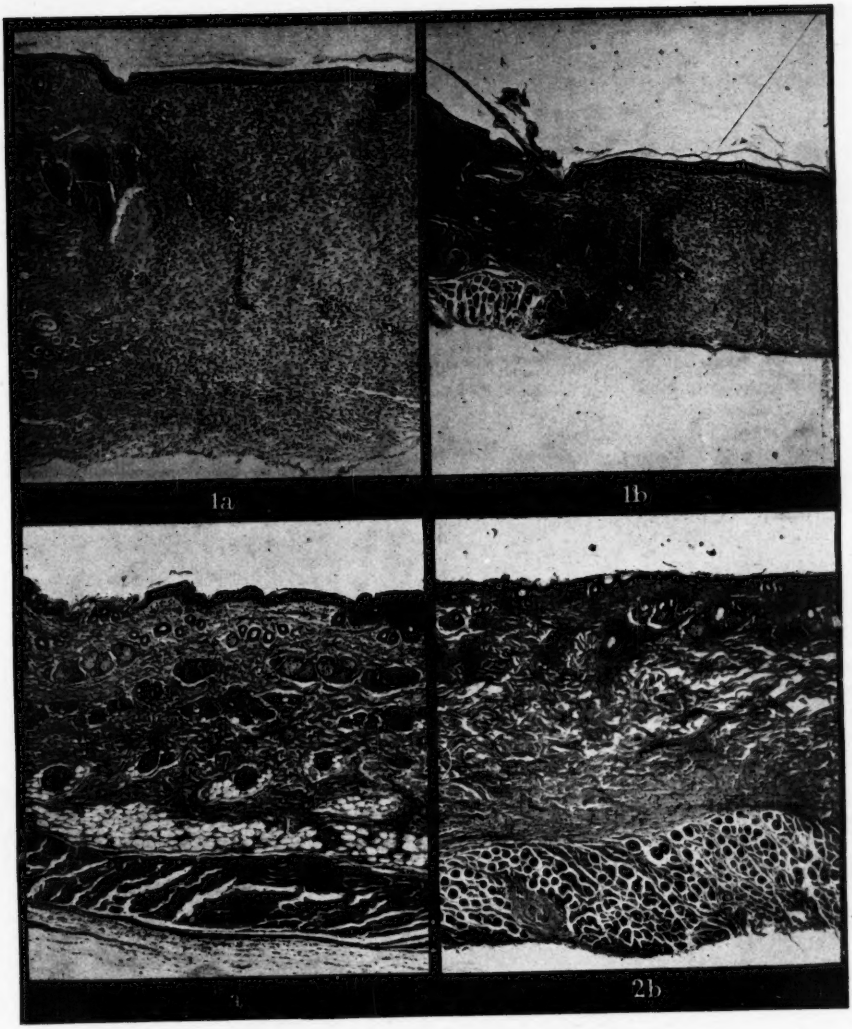
FIG. 3*b*.—Rabbit. Control. The same wound as in Fig. 3*a* photographed 10 days later.

FIG. 3*c*.—Rabbit. Cortisone-treated. This rabbit was wounded on the same day as the control, but received cortisone. Appearance of wound on the 16th day.

FIG. 3*d*.—Rabbit. Cortisone-treated. The same wound as in Fig. 3*c* photographed 10 days later.

FIG. 4*a*.—Rabbit. Control. Transverse section of the ear 13 days after wounding. Note the abundant granulation tissue. H. and E. × 45.

FIG. 4*b*.—Rabbit. Cortisone-treated. Transverse section of the ear 13 days after wounding. Compare the thickness of granulation tissue with that in Fig. 4*a*. H. and E. × 45.


1a
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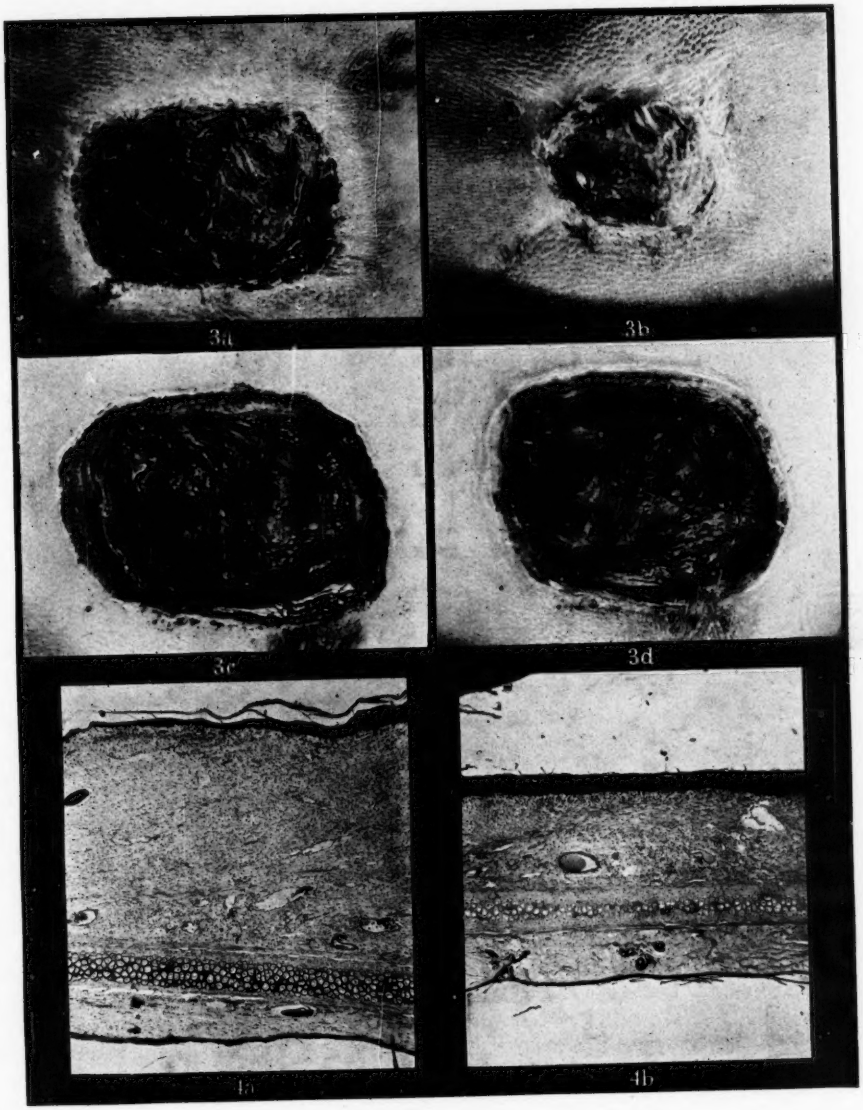
3a
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narrowing, and in some animals a loss of the panniculus adiposus and repression of hair-follicle growth. There was no evidence of any gross changes in the amount or distribution of elastic fibres, but the collagen fibres appeared more compact (Fig. 2*a* and *b*).

B. *The Effect of Cortisone on the Healing of Skin Wounds in Guinea-pigs.*

Young male guinea-pigs weighing 200–250 g. were divided into two groups of 8 each. Group I received 2·5 mg. of cortisone intramuscularly (approx. 12·5 mg./kg. body-weight) daily throughout the experiment. Group II received an equivalent volume of saline. The animals were anaesthetized 24 hours after the first injections, and 4 equally-sized wounds made in the skin, using the technique already described. Both groups of guinea-pigs were fed liberally on Parkes No. 41 diet with fresh cabbage daily.

On days 3, 5 and 9 two animals, and on days 11 and 13 one animal from each group were killed and the skin wounds examined histologically.

Results.—In this series the healing time of each wound was recorded, the criterion for a fully healed wound being an intact epithelial surface with no adherent scab. At the dose level of cortisone used, no difference in the healing time was observed between the two groups of animals. Histologically the wounds from the cortisone-treated animals were similar in all respects to those in the control animals.

C. *The Effect of Cortisone on the Healing of Wounds in Rabbits.*

Two groups of 3 rabbits each were used. Group I received cortisone (7·5 mg./kg. body-weight daily) by intramuscular injection throughout the experiment and the controls received injections of saline. Three days after the injections had started each rabbit was anaesthetized and wounded. The wounds consisted of 5-mm. punch wounds, a large resection of skin of approximately 4 sq. cm. and the removal of 1 sq. cm. of skin from the surface of the ear. One control and one experimental animal were killed on the 4th, 8th and 13th day after wounding and the lesions were examined histologically. The rabbits were fed on Parkes No. 18 diet supplemented with cabbage three times a week.

Results.—From a day-to-day examination of the wounds it was obvious that the cortisone-treated animals were healing differently from the controls. By the 16th day the large wounds in the control animals showed some contraction and filling with granulation tissue; the cortisone-treated animals showed complete absence of any sort of response (Fig. 3*a–d*). The ear wounds showed similar changes up to the 8th–9th day, but by the 13th day a small amount of granulation tissue had appeared and the epithelium had regenerated and covered the wound. Histologically the granulation tissue in the cortisone-treated animal did not differ qualitatively from that of the control, the most striking difference being quantitative. All the elements of normal granulation tissue were present in a quantity proportionate to the amount of new tissue that had formed. In other words, neither the inflammatory cellular elements nor the capillaries, fibroblasts and intercellular ground substances showed any gross abnormality (Fig. 4*a* and *b*). This finding is contrary to that of Ragan, who likened the granulation tissue in cortisone-treated animals to that found in animals rendered scorbutic.

DISCUSSION.

The changes in the skin of the rats treated parenterally with cortisone are very similar to those reported by Baker, Ingle, Li and Evans (1948). These authors administered ACTH to their rats, and found that after a period of some 10 days' treatment there occurred thinning of the skin, a reduced growth of hair and a reduction in the size of the panniculus adiposus. These changes were also accompanied by general loss of weight. ACTH has also been shown by Evans, Simpson and Li (1943) to inhibit general body growth, and even to antagonize the action of pituitary growth hormone. Marx, Simpson, Li and Evans (1943), Baker and Ingle, Li and Evans (1948), in discussing these findings, suggested that the existence of protein catabolism and an increase in the excretion of urinary nitrogen proportional to the loss in body weight was the most probable explanation for the inhibition of growth. Baker and Whitaker (1948), however, in a later paper were able to show that cortisone could produce involution of epidermal elements without catabolism of liver proteins, presumably without the existence of a negative nitrogen balance. In their experiments cortisone was applied locally to the skin and only the treated areas showed involutionary changes. These changes correspond well with the observations of Baker and Whitaker (1950), who delayed the healing of wounds and the formation of granulation tissue with a locally applied suprarenal extract. There is, therefore, a strong case for suspecting that cortisone inhibits wound healing either by antagonizing pituitary growth hormone or by retarding cell growth directly.

Another aspect of the problem is the effect of cortisone on the development of acute inflammatory responses. Selye (1949) injected formalin into the tissues, and thereby produced acute and subsequently chronic inflammatory changes at the site of inoculation. Cortisone reduced or abolished the inflammatory response, and so protected the animal from the development of a granuloma and its extensive scar. In this sort of experiment, cortisone may be reducing the amount of granulation tissue by subduing the initial inflammatory response. Carrel (1930) has indeed suggested that growth-activating substances are liberated by leucocytic and other ferments acting on cell débris and fibrin—in other words, just those substances that are known to be produced in inflamed tissues and which cortisone seems able to reduce.

There remain two further possibilities, both of which might reduce hyperaemia —altered capillary permeability and the formation of fibrin. Firstly, cortisone may be preventing the formation of chemical stimulators, e.g. leukotaxin and H substance or histamine, or secondly the surviving cells may be made insensitive to these substances by cortisone.

That suprarenal hormones play a significant role in the regulation of fluid exchange as manifested by capillary filtration has long been suspected. Menkin (1940), for instance, showed that the effect of inflammatory exudates or of leukotaxin in increasing capillary permeability was wholly or partly inhibited by the presence of an extract of suprarenal cortex.

In order to test this latter hypothesis an investigation was made to determine the influence of cortisone on the action of solutions of leucotactic peptides, and of histamine in increasing skin capillary permeability.

PART II.

The Effects of Cortisone on Histamine Skin Responses and on Intracutaneous Injection of Leucotactic Peptides.

The leucotactic peptides were prepared by Dr. W. G. Spector, working in this department. They are made by de-proteinizing a peptic digest of fibrin, and in the dry state remain active for many months.

The dilutions of leucotactic peptides were made up immediately before use, 10 mg. being dissolved in 1·5 ml. of 0·9 per cent NaCl and 10-fold dilutions made from the 1/100 stock. The histamine dilutions were also made up in 0·9 per cent NaCl.

The rabbits were anaesthetized with Nembutal and their abdomens carefully depilated with a depilatory paste. After a thorough washing with warm water the rabbits were placed on their backs on top of a warm water bath, where they remained throughout the experiment. Trypan blue (Gurr), 3·5 ml./kg. body-weight of a 2½ per cent solution, was then injected intravenously, and after waiting about 20 min. to make sure that there were no inherent blue areas, the dilutions of luecotactic peptides and histamine were injected. The intracutaneous injections were limited to a volume of 0·1 ml., and were made with a tuberculin syringe and a fine needle.

Readings were made 30 minutes after injection, and the colour intensities of the control and experimental animals compared and awarded arbitrary units, thus: +++, ++, +, 0. In each experiment two rabbits were used, one receiving an intramuscular injection of cortisone (10 mg./kg. body-weight) 18–24 hours before testing, while the controls received an equivalent volume of saline. Three weeks later the same two rabbits were again tested, the original control now receiving the cortisone.

In each of these experiments it was noticed that the areas that had been manipulated by the left hand, the right holding the syringe, gradually assumed a blue colour due presumably to localized injury and increased capillary permeability and leakage of dye. The intensity of staining of these areas in the control and experimental animals was recorded and appears with the other results in Table I. These results show that cortisone given 18–24 hours before testing reduced the amount of dye leakage following the intracutaneous injections of histamine and of leucotactic peptides. Moreover, the staining that results from the manipulation of the skin was also reduced.

In an effort to overcome animal-to-animal variation and to find out how soon cortisone was effective, a further series of rabbits was anaesthetized, depilated and injected with leucotactic peptides and histamine. Cortisone was then injected and the substances were re-tested at intervals of 1, 2 and 3 hours. In this way each rabbit acted as its own control, the activity of the solutions being checked by including an untreated animal.

The results of this series of experiments appear in Table II. Here again, and within two hours of an intramuscular injection, cortisone prevented the leakage of dye into the tissues.

DISCUSSION.

It is tempting to correlate the subduing effects of cortisone on the formation of granulation tissue with its apparent ability to protect small blood vessels from

TABLE I.—*The Degree of Staining at the Site of Intracutaneous Injections of Histamine and Leucotactic Peptides in Rabbits Injected Intravenously with Trypan Blue.*

Experiment number.	Rabbit number.	Control. Leucotaxin. 1/100.	1/1000.	1/10,000.	Histamine. 1/1000.	1/10,000.	1/100,000.	Background.	Rabbit number.	Cortisone pre-treated. Leucotaxin. 1/100.	1/1000.	1/10,000.	Histamine. 1/1000.	1/10,000.	1/100,000.	Background.
1	1	+++	++	++	+++	+++	++	+++	2	0	0	0	0	0	0	0
2	3	++	++	+	+++	+++	++	+	4	+	+	0	0	+	0	+
3	5	+++	++	+	+++	++	+	++	6	+	+	0	0	0	0	0
4	7	++	++	+	++	+	0	++	8	++	0	0	0	..	..	0
						3 weeks later.										
1	2	+++	++	+	+++	+++	++	++	1	+	+	0	+	0	0	+
2	4	+++	++	+	++	++	+	++	3	++	0	0	0	+ 0		0
3	6	+++	+++	++	+++	+++	++	+	5	+	0	0	+	0	0	0
4	8	+++	++	+	+++	++	+	++	7	+	++	0	0	0	0	0

TABLE II.—*The Degree of Staining at the Site of Intracutaneous Injection of Histamine and Leucotactic Peptides in Rabbits Injected with Trypan Blue, 1 and 3 Hours after a Single Intramuscular Injection of Cortisone.*

Rabbit number.	Initial. Leucotaxin. 1/100.	1/1000.	1/10,000.	Histamine. 1/1000.	1/10,000.	1/100,000.	1 hour. Leucotaxin. 1/100.	1/1000.	1/10,000.	Histamine. 1/1000.	1/10,000.	1/100,000.	3 hours. Leucotaxin. 1/100.	1/1000.	1/10,000.	Histamine. 1/1000.	1/10,000.	1/100,000.
							Control.											
9	+++	++	+	++	++	++	+++	++	+	++	++	++	+++	++	+	++	++	++
10	+++	++	+	++	+	+	+++	++	+	++	+	+	No record.					
							Cortisone.											
11	+++	++	0	+++	++	0	++	++	0	0	0	+	+	0	0	0	0	0
12	+++	++	0	+++++		+	+++	+	0	++	0	0	+	0	0	0	0	0
13	+++	+	0	++	++	0	++	+	0	++	++	0	+	0	0	0	0	0

substances capable of increasing their permeability. Inflammation and repair can be regarded as a sequence of reactions, each reaction dependent both qualitatively and quantitatively upon its predecessor. If, as seems likely in the case of wound healing in rabbits, cortisone is merely depressing a normal process, its action is most probably that of controlling the degree of reactivity, rather than altering its actual pattern. Whether cortisone exerts its effect throughout the process of wound healing is not known, but it is evident from the results obtained that cortisone is active in its earliest stages, namely, the stages of hyperaemia and oedema formation. It is not surprising therefore to find that cortisone is capable of inhibiting a variety of allergic skin responses, all of which are thought to depend ultimately upon the local release of histamine or of active leucotactic peptides.

A hypothesis, therefore, that cortisone acts by reducing the sensitivity of surviving tissue to the prime movers of the inflammatory response lends itself to an interpretation of the known effects of cortisone in the treatment of rheumatic fever and rheumatoid arthritis, both conditions representing an exaggerated inflammatory response. Furthermore, the report by D'Arcy Hart and Rees (1950) that a quiescent form of tuberculosis in mice flares up when cortisone is given, supports the view that the inflammatory reaction is being subdued, in this instance to the detriment of the animal.

SUMMARY.

1. The effects of cortisone on the healing of skin wounds in various animals have been studied. Cortisone has no effect on wound healing in guinea-pigs ; it probably reduces the amount of granulation tissue formed by rats, and retards wound healing in rabbits.

2. Solutions of leucotactic peptides and of histamine have been injected into the skin of rabbits, and the effect of cortisone on the formation of the wheals has been observed.

3. These experiments confirm the results of other workers, and show that at a relatively high dose level in rabbits the process of wound healing may be retarded. The demonstration that cortisone may inhibit the skin reactions to histamine and leucotatic peptides suggests a mechanism whereby inflammatory and the subsequent reparative processes are subdued.

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THE EFFECTS OF BIOCHEMICAL MUTATION ON THE VIRULENCE OF *BACTERIUM TYPHOSUM*: THE LOSS OF VIRULENCE OF CERTAIN MUTANTS.

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PREVIOUS papers (Bacon, Burrows and Yates, 1950*a* and *b*) reported the isolation and characterization of a number of biochemical mutants of *Bacterium typhosum* and recorded their virulence for mice. Whilst the majority of mutants remained unaltered in virulence, a number of specific mutations resulted in a marked decrease, the virulence of purine mutants in particular being of a very low order. The experiments reported here were designed to investigate possible reasons for the loss of virulence associated with these specific mutations.

MATERIALS AND METHODS.

Strains.

Five strains deficient in their ability to synthesize purines (479, 20080, 20092, 20095 and 20102), one (20063) requiring *p*-aminobenzoic acid (PABA), three (8505, 19341, 19662) requiring aspartic acid (" aspartic-less ") and three anomalous strains (9749, 20085, requiring histidine and 20058, requiring cystine) were investigated. Strains 479, 20063 and 8505 received most attention, as representatives of the three growth factor types with which marked loss of virulence was invariably associated.

Suspensions.

For convenience in obtaining the large numbers of relatively avirulent organisms required for lethal doses, cultures grown for 16 hours on tryptic meat digest agar (T.M.A.) were employed in place of cultures on growth factor supplemented minimal medium. In all other respects the details of preparation of suspensions, media and buffer and methods of conducting virulence tests are as previously described (Bacon, *et al.*, 1950*a* and *b*).

EXPERIMENTAL AND RESULTS.

In common with other purine-less mutants, 479, as previously noted, conforms with the accepted criteria defining virulent strains of *Bact. typhosum*. It grows readily on T.M.A., producing smooth, circular, more or less opaque colonies indistinguishable from those of the virulent strain Ty22 from which it was derived. It is morphologically identical with Ty22, and equally sensitive to lysis by specific Vi phage. Both mutant and parent are freely motile from growths on T.M.A. and non-motile when grown on minimal medium. They both resist O-aggluti-

nation in the living state, and possess Vi and O somatic antigens as determined by agglutination of living suspensions by Vi antisera, and of heat-killed suspensions by O antisera. No qualitative differences in their agglutinogenic structure were revealed by reciprocal absorption tests with their antisera. Quantitatively, 479 is slightly more O-agglutinable than the parent, suggesting a lower complement of Vi antigen, this being confirmed by comparative quantitative immunization experiments in rabbits using alcoholized vaccines (Felix, 1941). It seemed unlikely, however, that the quantitative difference in the abundance of Vi antigen in this mutant was responsible for the low virulence observed, in view of the fact that its O-agglutinability was of the same order or less than that of a number of other mutants of high virulence. Further, mutant 20080, which is as resistant as Ty22 to O-agglutination, is of the low virulence shown by 479, and mutants 479 and 20080 are of lower virulence than the classical strain 0901, which is completely devoid of Vi antigen. The toxicities of 479 and Ty22 suspensions killed by heating at 60° C. for 30 minutes are of the same order.

Comparative survival of mutant 479 *and Ty*22 *in mice.*

Although mutant 479 was known to be recoverable by culturing the spleens of mice seven days after injection with 500 million organisms, and therefore was able to survive in the host for this period, it was considered of interest to compare its survival with that of the virulent Ty22. The nutritional difference existing between the two strains permitted the enumeration of each in mixed suspensions, and allowed the survival rates of the two strains to be determined in the same animal, and therefore under identical environmental conditions. Mice were injected with a mixed suspension containing 1 million cells of 479 and 1 million of Ty22. At intervals of two days after injection pairs of mice were killed by cervical dislocation, their spleens removed, macerated in a blendor, serially diluted and plated on minimal medium supplemented with adenine to a final concentration of 2μM. On such a medium mutant 479 produced very small colonies readily differentiated from those of Ty22. The mutant in these experiments was found to be eliminated from the host considerably more rapidly than the parent. Zelle (1942), investigating the relative survival of colonially distinguishable strains of *Salmonella typhi murium*, similarly showed the rough avirulent strains to be less well suited to survival in the host than the virulent smooth strains.

We considered this possibly reflected a differential resistance to phagocytosis between 479 and Ty22. *In vitro* tests by the method of Bhatnagar (1935) using rabbit or mouse leucocytes showed the two strains to be phagocytosed equally and at a considerably lower rate than strain 0901.

Phagocytosis *in vivo* was investigated in the mouse as follows: Phagocytes were mobilized to the peritonea of a series of mice by the injection of doses of 1 ml. of 2 per cent (w/v) wheat starch in saline intraperitoneally. After 16 hours 500 million Ty22 or an equal number of 479 cells were injected by the same route, and pairs of mice sacrificed after 10, 15, 20, 30, 60, 120, 180 and 240 minutes. Films were prepared of the peritoneal exudate, stained (Leishman) and the phagocytic indices of the two strains determined. From 120 minutes to the termination of the experiments estimates of the numbers of phagocytes present in the peritoneum were made, using a haemacytometer, and after 4 hours

the numbers of free organisms were determined by plate counts of the peritoneal washings. The latter counts would include ingested organisms capable of producing colonies on agar.

Over the initial 2-hour period following injection the phagocytic indices of the two strains were similar. Subsequently the numbers of Ty22 ingested exceeded those of 479. It was very evident, however, from the films prepared that the background of uningested organisms in the Ty22 series progressively increased with time, whilst in the 479 series the converse held, so that after 4 hours the phagocytes themselves exceeded the numbers of uningested 479 cells. The injected dose of Ty22 had in this time increased to give 2230 million free organisms, and the same dose of 479 decreased to 13 million. The numbers of phagocytes with ingested organisms in the Ty22 series was then 26 million, and in the 479 series 45 million, the latter figure indicating that few, if any, ingested organisms were capable of producing colonies on agar. The loss of virulence of the mutant therefore appeared to result from its inability to outstrip phagocytosis by division, suggesting sensitivity to an inhibitory agent present in peritoneal fluid, ineffective against Ty22, or that the mutant was unable to multiply owing to the absence of specific growth factors from the environment. The peritoneal fluid was therefore tested for the presence of available growth factors for 479, and for a random selection of virulent and avirulent mutants.

Availability of specific growth factors in the mouse.

The peritoneal cavities of 30 mice were each rinsed thoroughly with 2 ml. citrated minimal medium using aseptic technique, the washings centrifuged free from cells and debris, incubated for sterility and pooled. To tubes of 6 ml. minimal medium, 1 ml. of washings was added and the tubes seeded with a small washed inoculum of the different mutants. Controls comprising minimal medium with and without specific growth factor and supplemented with both peritoneal fluid and growth factor were included for each mutant. Growth was recorded daily by plate counts or turbidimetrically.

The data obtained for mutant 479 and the virulent mutant 10020 requiring pantothenate are recorded in Table I, and the complete results summarized in Table II.

It is clear from Table II that the peritoneal fluid of normal mice contains an abundance of those growth factors required by mutants showing a high virulence. It is equally clear that those required by purine- or PABA-less mutants are of limited availability, and could well account for the low virulence shown by them. The rapid and abundant growth obtained in minimal medium supplemented with both peritoneal fluid and growth factor indicated the absence of any inhibitory substance, effective against mutants of low virulence, at the dilution of peritoneal fluid employed.

An examination of the distribution of viable organisms in mice moribund from an infection of Ty22 injected intraperitoneally showed the vast majority to be localized in the peritoneal cavity. Here the organisms were approx. 1000 times more abundant than the total in the blood stream, and considerably exceeded the sum of those recoverable from all other parts of the animal. It is reasonable to assume, therefore, in the absence of information to the contrary, that the vast numbers developing in the peritoneum are mainly responsible for the pathological

TABLE I.—*Determination of Available Growth Factors for Mutants in Mouse Peritoneal Fluid.*

Mutant.	Minimal medium supplemented with—	Growth: Organisms per ml. × 10^{-6}. Days' incubation. 0.	1.	3.	5.	Organisms supported by growth factor available per peritoneum × 10^{-6}.
479	..	0·003	0·004	0·0004	0	
	Adenine + aneurin	0·003	0·007	100	1550	
	Peritoneal fluid	0·002	0·002	0·0001	0	0
	Adenine + aneurin + peritoneal fluid	0·003	750	1350	1550	
10020	..	0·002	0·005	0·002	0	
	Pantothenate	0·004	0·015	820	1530	
	Peritoneal fluid	0·004	550	660	730	10,200
	Pantothenate + peritoneal fluid	0·003	900	1250	1530	

TABLE II.—*Numbers of Mutant Organisms Supported by the Available Growth Factors in the Peritoneal Fluid of a Mouse.*

	Mutant.	Growth factor.	Organisms × 10^{-6}.
Mutants of high virulence	8974	Nicotinamide	15,000
	20039	Aneurin	14,400
	20022	Phenylalanine	13,900
	10020	Pantothenate	10,200
	9355	Valine + isoleucine	8,400
	5459	Methionine	6,300
	20057	Cytosine	6,300
Mutants of lowered virulence	6346	Histidine	3,000
	20082	Leucine	1,800
	20034	Glycine	1,800
	8505	Aspartic acid	1,200
	20063	PABA	0·56
	20080	Purine	0·0
	479	Purine + aneurin	0·0

condition, and that a study of the conditions for growth in the peritoneum is pertinent to a study of typhoid infection in mice injected by the intraperitoneal route.

Recovery of virulence by injection of specific growth factors.

If the loss of virulence of purine- and PABA-less mutants is attributable to the limited availability of specific growth factor from the peritoneal exudates of the host, it should be possible to recover the virulence of the parent type by injecting

with the mutant a suitable quantity of its growth factor. The high toxicity of adenine to mice prevented the addition of more than 2·0 mg. of this purine. A dose of 0·5 mg. injected with 479 suspensions resulted in a two-fold increase in virulence. Prolongation of the effective time over which the growth factor was available by the injection of 0·17 mg. adenine with the organisms, followed by two similar doses at 2 and 4 hours later, resulted in a four-fold increase in virulence. The much lower toxicity of hypoxanthine permitted growth factor additions of at least 8 mg. per dose. Graded doses of this purine resulted in the enhancement of the virulence of 479 to a maximum equal to that shown by Ty22 in the presence of hypoxanthine, as illustrated in Fig. 1. Hypoxanthine alone

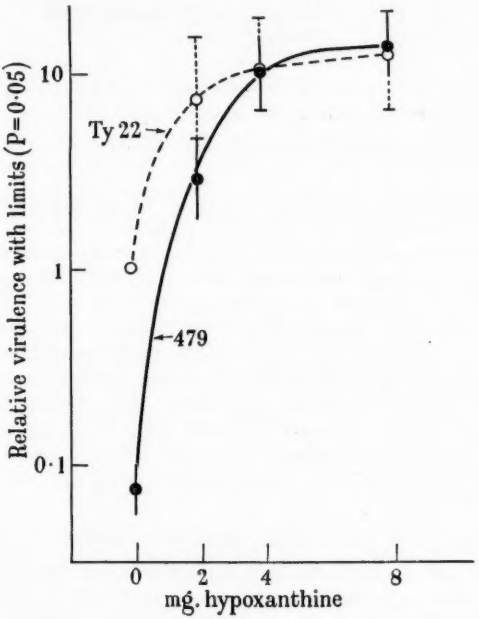

FIG. 1.

was without apparent effect on mice in doses of 16 mg. The low solubility of this purine in buffer necessitated its administration as a fine suspension, which probably enhanced the effect by retarding its elimination from the peritoneum.

In a similar manner the virulence of PABA-less mutant 20063 was raised to the level of Ty22 by the injection of 2 mg. PABA suspended in arachis oil. In experiments of this nature it is necessary to prolong the existence of the added growth factor in the peritoneum, until a lethal dose of organisms has had time to develop from the injected dose. With the progress of time the demands of the organisms for growth factor probably increase logarithmically, whilst the injected growth factor is being eliminated at an unknown, but probably rapid, rate. A considerable excess of growth factor must therefore be provided initially to ensure

its presence in the later stages of infection, or the factor must be injected with a vehicle (e.g. arachis oil) permitting its slow liberation during the infection. Doses of 2·5 mg. PABA dissolved in buffer had only a slight virulence-enhancing effect on mutant 20063, presumably owing to very rapid elimination from the peritoneum. The specificity of the injected growth factors in enhancing the virulence of purine and PABA-less mutants is illustrated in Table III.

TABLE III.—*Specificity of Injected Growth Factor in Enhancing Virulence of Purine-less and of PABA-less Mutants.*

Organism.	Deaths out of 20 mice injected with 4 mg.:—		
	—	hypoxanthine	PABA
—	0	0	0
Ty22	13	18	11
479	0	16	0
20063	0	1	16

Dose: 20 million organisms. Growth factors suspended in 0·25 ml. arachis oil.

For full growth in minimal medium aspartic-less mutants require the addition of L-aspartic acid to a level of 1000μM as compared with 40–100μM levels required by the majority of other amino-acid- or purine-requiring mutants. It is probable therefore that large amounts of growth factor would initially be necessary to ensure the presence of sufficient quantities in the later stage of infection of mice with aspartic-less mutants. Doses of 6 mg. of aspartic acid suspended in arachis oil, whilst having no effect on the virulence of Ty22, considerably enhanced the virulence of the aspartic-less mutant 8505, but did not result in full enhancement to the level shown by the non-exacting parent. Injection of larger amounts of aspartic acid or of calcium aspartate showed some toxicity to the test animals and could not be employed. Aspartic acid injected in doses of 6 mg. resulted in reduction of the average lethal dose of mutant 8505 from 122 millions to 29 millions.

Reversion of mutants to the non-exacting state and its effect on their virulence.

Washed inocula of mutants of low virulence were heavily seeded (approx. 2×10^8 cells per plate) on minimal agar and incubated. Cells reverting to the non-exacting condition are capable of growth on this medium, and recognizable as large colonies against the background film of mutant cells. Large colonies which developed were isolated and confirmed as typhoid organisms by phage testing, and for their ability to grow in minimal medium without growth factors. By this means we obtained reverted strains of 479, 20092, 20063, 19662, 9749 and 20058. We have not succeeded in obtaining similar reversions of mutants 20080, 20095, 20102, 19341, 8505 or 20085 despite repeated attempts at their isolation, involving the plating of over 2×10^9 organisms in several experiments. It is of interest to record that mutant 479 reverted to the loss of its requirement for a purine and for aneurin in a single step, and no back mutations to separate loss of one or other requirement have been isolated.

Table IV records the virulence of the reverted mutants obtained.

It is seen that biochemical mutants which have no accompanying morphological or colonial variation recovered a virulence comparable with that of the

TABLE IV.—*Change in Virulence of Mutants on Reversion from Growth Factor Dependence to Independence.*

Mutant.	Relative virulence with limits (Ty22 = 1·0, P = 0·05).	
	When dependent.	When independent.
479	0·04 (0·03–0·05)	0·70 (0·49–1·0)
20092	0·03 (0·01–0·04)	0·55 (0·33–0·88)
19662	0·14 (0·09–0·2)	0·70 (0·4–1·0)
20063	0·04 (0·03–0·06)	1·1 (·78–1·6)
9749	0·05 (0·03–0·07)	0·10 (0·06–0·16)
20058	0·02 (0·01–0·03)	0·06 (0·04–0·09)

parent on reversion to the non-exacting condition, suggesting that the biochemical mutation was responsible for the observed loss of virulence. Biochemical mutants 9749 and 20058 are also morphological variants. These retain their low virulence and their morphological variation when reverted to independence of added histidine or cystine respectively. The loss of virulence and morphological variation shown by these mutants evidently is not attributable to biochemical mutation.

Variation in virulence.

Throughout this work, conducted over a period of several months, it was found that the relative virulence with respect to Ty22 of mutants of lowered virulence was not constant. Virulence assays repeated at later dates did not confirm those made several months or even weeks previously. The average lethal dose of mutant 479, for example, in June was 3300 million organisms (relative virulence to Ty22 0·009). This number decreased with reasonable regularity to a minimum of 232 (relative virulence 0·09) by September, and subsequently increased. The average lethal dose of mutant 20063 varied erratically between extremes of 1025 million (relative virulence 0·015), and 176 million (relative virulence 0·10). In the light of the experiments reported in this paper it is suggested that the variation in virulence reflects a seasonal or otherwise variation in the composition of mouse peritoneal fluid. Support is given to this view by the fact that in a repeat assay of available purines in mouse peritoneal washings, at the time of highest virulence of mutant 479, this mutant was able to develop to a level of 210 million (cf. Table II), as opposed to no growth in the original assays made when 479 was at its lowest virulence.

The average lethal dose of the standard Ty22 also varied over the period of these experiments, particularly during the period May to July. In experiments conducted at 3–5-day intervals the average lethal dose of 13 million on May 3 became successively 16, 29, 40, 42, 60, to reach a maximum of 73 million on June 9, and subsequently declined 43, 33, 27 to 22 million by July 6.

Effect of dietary growth factors on virulence of mutants.

It was considered of interest to investigate whether, by incorporating specific growth factors in the diet of our experimental animals, we would be able to raise their availability in the host and thereby specifically increase the virulence of our

mutants. Mice maintained on a normal diet but fed 10 mg. hypoxanthine in their drinking-water daily for 4 days prior to challenge with mutant 479 showed no decrease in resistance to infection. Similar negative results were obtained with mice ingesting 20 mg. asparagine daily for the same time, and challenged with mutant 8505. However, it was possible considerably to enhance the virulence of mutant 20063 by the incorporation of small amounts of PABA in the diet, as illustrated in Table V. The results indicate a slight cumulative effect of dietary PABA on the available concentration of this growth factor in the mouse. The incorporation of 10 mg. PABA in the diet on 5, 3 and 1 days prior to challenge permitted mutant 20063 to show a virulence equal to that of Ty22.

TABLE V.—*Effect of Dietary PABA on the Virulence of Mutant* 20063.

Culture.	PABA given on days—					Challenge. Dose × 10^{-6}.	Deaths.
	5.	4.	3.	2.	1.		
20063	—	—	—	—	—	400	3
	—	—	—	—	—	100	0
	—	—	—	—	+	100	16
	—	—	+	+	+	100	18
	+	+	+	+	+	100	20
Ty22	—	—	—	—	—	40	12
	—	—	—	—	—	10	8
	—	—	—	—	+	10	9
	—	—	+	+	+	10	10
	+	+	+	+	+	10	3
—	+	+	+	+	+	—	0

20 mice per treatment, each given 2 ml. water or PABA solution (5 mg. per ml.) on the days prior to challenge as indicated. + = PABA ; — = water.

Variation in the amount of PABA in the diet of our test animals (which until received by us was not within our control) could well explain the variation in virulence experienced with mutant 20063. It is probable that factors other than dietary sufficiency control the level of available purines in the mouse, since mammals are independent of an exogenous supply of these compounds (Benedict, 1917).

Synergistic action of mutants.

If the low virulence of purine- and PABA-requiring mutants is due to a limitation of available growth factor preventing their development to a level toxic to the host, when two mutants having different growth factor requirements are simultaneously injected each theoretically should grow to the limits of the available factors, and the total number of organisms developing should equal the sum of each separately. That is, a double infection with two such mutants would be expected to be more lethal than either separately. This was found to be the case, as illustrated in Table VI. Mutants of high virulence, e.g., the nicotinamide-less 20035 and phenylalanine-less 20022, injected together were no more virulent than either separately ; neither were two low virulence mutants having the same growth factor requirement (mutants 479 and 20102).

TABLE VI.—*Synergistic Effect of Purine and PABA-less Mutants.*

Total dose of organisms $\times 10^{-8}$.	Deaths out of 20 mice injected with—		
	479.	20063.	479 + 20063.
100	0	0	2
200	0	1	17
400	2	1	18
800	9	10	20

Although the injected suspension of 479 plus 20063 contained approximately equal numbers of each mutant, and the virulence of these mutants was at the time of these experiments of the same order, about 30 times as many PABA- than purine-requiring organisms were recoverable from mice moribund from the double infection. Mutant 479, like Ty22, when grown in minimal medium plus growth factor liberates into the medium effectively large amounts of growth factor active for our PABA-requiring mutant. 20063 similarly grown, like Ty22, liberates effectively small amounts of growth factors supporting purine-requiring mutants. It is probable, therefore, that in a mixed infection as described above the PABA-less mutant obtains a considerable amount of its specific growth factor from over-production by mutant 479. Such an effect is readily demonstrable *in vitro* by growing mutants 479 and 20063 in media containing suboptimal amounts of PABA, purines and aneurin, and observing the total growth occurring in tubes inoculated with each mutant separately and with both together.

DISCUSSION.

Biochemical mutation to particular growth factor dependence in *Bact. typhosum* has clearly been shown to modify considerably the virulence of this organism. Those growth factors required by mutants showing a high virulence are abundant in mouse peritoneal fluid, whereas purines, *p*-aminobenzoic acid and aspartic acid are of limited availability. Following intraperitoneal inoculation, the vast majority of viable organisms in mice moribund from the infection are to be found in the peritoneal cavity. The addition of purines, PABA or aspartic acid to the peritoneum specifically enhances the virulence of mutants dependent on the presence of these factors for growth ; and the reversion of these mutants to independence of these factors results in full recovery of their virulence. These observations are consistent with the view that loss of virulence of purine- PABA- or aspartic acid-requiring mutants results from their inability to develop from a sublethal inoculum to a level fatal to the host because of insufficient availability of their specific growth factors in the host.

The low virulence of the morphologically altered mutants 9749 and 20058 is independent of their biochemical mutation to requirements for histidine or cystine. On reversion to the non-exacting state they retain their low virulence and morphological variation. These mutants are therefore not at variance with the statement, based on the evidence of 8 other histidine-less and 10 other cystine-less mutants, that mutation to these growth factor requirements *per se* has little effect on the virulence of mutants of these types.

With our *p*-aminobenzoic acid dependent mutant 20063 we have been able to give a clear demonstration of the effect of specific dietary constituents on the resistance of mice to infection with this mutant, and to afford an explanation for the variation in virulence of the strain observed over a period. The increased resistance to infection with avian malaria parasites shown by pantothenic acid- or riboflavin-deficient chicks in the experiments of Brackett, Waletsky and Baker (1946) and of Seeler and Ott (1944) by analogy may be indicative of the growth factor dependence of these protozoa on these vitamins.

Our growth factor feeding experiments imply that whereas the mouse finds it necessary to maintain a constant low level of available purines for any given set of environmental conditions, the level of available PABA is not so critical, and accumulation of this factor can readily be achieved. The physiological conditions governing the availability of purines are unknown. As judged by their effect on the virulence of purine-requiring mutants, dietary purines are apparently not absorbed, or are made unavailable to such mutants very rapidly after absorption. Experiments of this nature emphasize the importance of maintaining experimental animals under constant dietary and environmental conditions throughout the whole course of an investigation on a host-parasite relationship, particularly where the parasite under investigation is an exacting organism.

The variation in virulence shown by suspensions of Ty22 prepared by a standard method from dried cultures also could be explicable on a basis of available nutrilites in the host. If it is assumed that the primary defence mechanism against typhoid infection in mice is phagocytic, then factors enhancing the growth rate of the organism would allow it the more easily to outstrip phagocytosis by division. Presumably the growth rate is controlled by the synthetic process which proceeds at the slowest rate. In a medium containing the majority of metabolites the growth rate will be governed by the rate of synthesis of those essential metabolites which are not available in a preformed condition. In the mouse both purines and PABA have been shown to be limiting factors, so that for growth these substances must be synthesized by Ty22, and the rate of their synthesis will limit the growth rate of the organism. Provision of these factors would therefore be expected to enhance the growth rate up to a maximum until the synthesis of some other essential metabolite becomes limiting. Our experiments have shown that increasing additions of both purines and PABA to the peritoneum increase the virulence of Ty22 up to a maximum, as would be expected on the above hypothesis. Purines are particularly effective in this respect, giving a 12-fold virulence enhancement of Ty22 in experiments conducted in September, when the behaviour of mutant 479 indicated the level of available purines in the mouse to be at a maximum. The 3-fold enhancement of the virulence of Ty22 given by PABA whilst purines are of limited availability could be due to the inter-relationship between purine and PABA synthesis suggested by the work of Snell and Mitchell (1942) and others. It is not unlikely that a particular nutrilite in addition to purines and PABA became limiting over the period of decrease in virulence of Ty22 between May and June, and subsequently returned to normal availability from June to July.

A clear example of the synergistic effect of two mutants of lowered virulence has been given in our experiments with mutants 479 and 20063. The effect most probably is attributable to reciprocal growth factor excretion by the two strains, allowing the development of each to a higher level in the host than would other-

wise have been possible. Similar synergism whereby organisms derive nutritional advantage from each other may well occur in some natural infections.

Where no serological or morphological differences are demonstrable between strains of pathogenic organisms of high and low virulence, this study emphasizes the necessity of conducting a close examination of the nutritional requirements of such strains to exclude a nutritional basis for the differences in virulence. It would, for example, be desirable to examine the nutritional requirements of the pathogenic and apathogenic strains of *Salmonella typhi-murium* described by Maaloe (1948) to confirm that the loss of invasiveness of the apathogenic strain did not result from a nutritional limitation. It is possible that the host and strain specificity of pathogenic organisms is in some cases determined by the nutritional requirements of the organism, a fundamental necessity for the manifestation of virulence in an exacting pathogen being the presence of adequate supplies of its essential growth factors in the host it is attempting to parasitize. Seasonal variation in the incidence of particular infections may from a similar cause be dependent on a seasonal variation in the abundance of available nutrilites in the host.

The production of particular nutritionally deficient mutant strains of pathogenic organisms provides a means of obtaining potentially fully virulent strains (prevented from manifesting their virulence owing to the limited availability of a specific growth factor from the host) to facilitate studies on a particular host-parasite relationship. The use of such strains as living vaccines is theoretically of immunogenic value, although involving a number of practical difficulties. Not the least would be the prevention of reversion to the non-exacting condition. The rate of spontaneous reversion could, however, greatly be reduced by incorporating a multiple genetic blockage in the synthesis of that factor whose deficiency results in loss of virulence, or by incorporating into one mutant a number of mutations in different synthetic systems leading to loss of virulence.

SUMMARY.

The loss of mouse virulence shown by mutants of *Bact. typhosum* with requirements for purines or for *p*-aminobenzoic acid is attributable to the limited availability of these specific growth factors in the host, preventing the development of such mutants to a toxic level. The full virulence shown by the parent type can be restored by the injection of specific growth factors into the host or by reversion of the mutants to independence of them. The low virulence of aspartic-less mutants is probably attributable to a similar cause.

The virulence of PABA-less, but not of purine- or aspartic-less mutants can be greatly enhanced by incorporating small quantities of specific growth factor in the diet of test animals. A variation in the virulence of mutants of *Bact. typhosum* experienced over several months is considered to reflect a seasonal or other variation in the availability of nutrilites in the host.

Mutants of low virulence exert a synergistic effect when injected as a mixed suspension into mice, provided such mutants have different growth factor requirements. Synergism probably results from the reciprocal excretion of specific growth factors by each type.

The low virulence of two anomalous strains having morphological, colonial or antigenic variation in addition to growth factor requirements was not attributable to their biochemical mutation.

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A PRESS FOR DISRUPTING BACTERIA AND OTHER MICRO-ORGANISMS.

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In the study of bacterial enzymes it is often of importance to extract the enzymes from the cells. Whilst some bacterial enzymes go readily into solution, others are firmly held by the insoluble cell structure and only leave the cell when the latter disintegrates. Owing to their smallness, bacteria are generally more difficult to disrupt than plant or animal tissues and no generally satisfactory method is as yet available. The present paper describes a press in which bacteria and other micro-organisms may be disrupted. The press consists of a cylinder in which the micro-organisms, together with abrasives, are subjected to sudden blows by a steel piston driven by a hand-operated mechanic's "fly-press." The piston forces the crushed material out of the cylinder into a reservoir. It is the suddenness of the pressure increase which causes the disruption, presumably owing to the shearing forces and penetrating effect of abrasive particles. The process takes 0·5 to 5·0 sec. to complete, and the material may be kept cold by pre-cooling the cylinder. Below — 20° no abrasive need be added because ice crystals act as abrasives.

A wide range of organisms has been crushed in the press. This includes *Clostridium welchii* and *Bacterium coli*, both of which are known to be relatively easily disrupted, as well as yeasts and certain *Mycobacteria*, which are considered to be difficult to disrupt (cf. Edson and Hunter, 1947). Compared with other available mechanical methods for disrupting bacteria (Wiggert, Utter, Silverman and Werkman, 1940; Kalnitsky, Utter and Werkman, 1945; Booth and Green, 1938; Curran and Evans, 1942; Muys, 1949; Milner, Lawrence and French, 1950), the press has the advantages that the time taken is short (0·5–5·0 sec.); that the material is kept cold (+ 2° to — 35°); and that it is unnecessary to dilute the cells by suspension in liquid media.

Some examples of the preparation of enzymes from the crushed material are given. Noteworthy among these are the preparation of fermentative extracts from *Cl. welchii*, formic hydrogenlyase from *Bact. coli* and fumarase from baker's yeast.

MATERIALS AND METHODS.

Description of the press.

General specification.—As shown in Fig. 1, the press consists of a stainless steel block formed from two rectangular pieces (A and B), each measuring 3.7/8 in by 1.7/8 in. by 5 in. (Firth Vickers 18-18 or S 60 are both suitable steels). The two sides are held in their correct positions by two placing pins (PP) and clamped

together by 4 stainless steel 3/8 in. Whitworth bolts ; these fit into channels (C) cut across the narrow section of the block. The common surfaces of A and B are machine ground and lapped with grinding paste. Two cylindrical holes, one 7/16 in. (B), one 11/16 in. (E) in diam., are bored from the upper surface of the block parallel to the ground surfaces, and with their centres on the division between

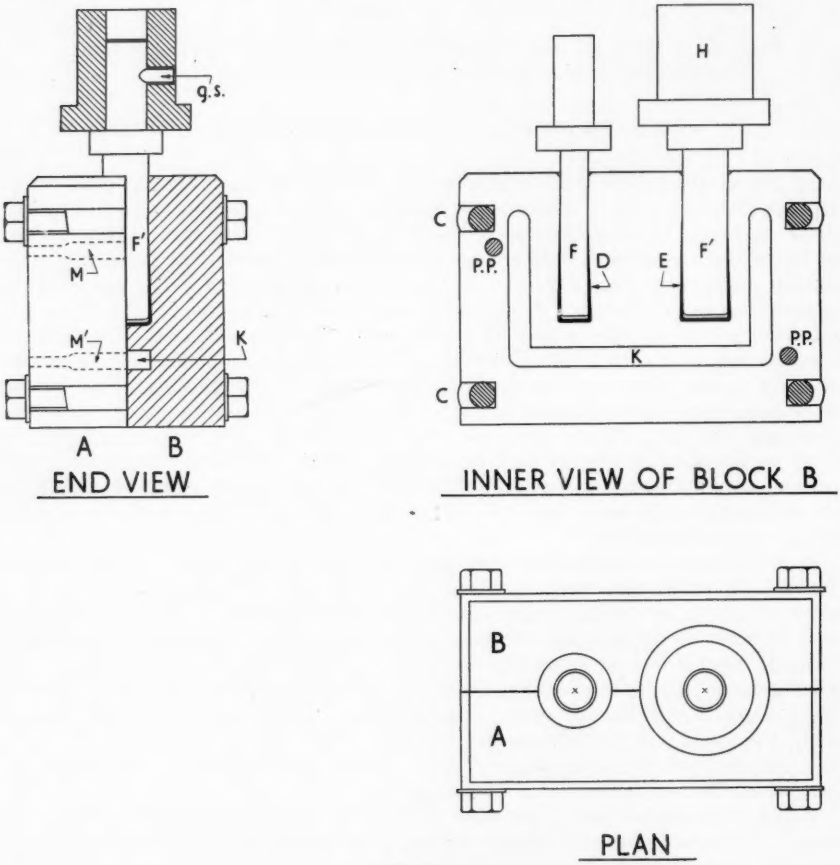


FIG. 1.

the two sides. Machine fitted pistons (F and F') (2.1/2 in. long) fit each of the holes. The tolerance of the pistons is 2/1000 to 3/1000 in. The pistons are interchangeable in an adapter (H), in which they are held by a grub (*gs*) screw. On the inner face of one side of the block a channel (K), 3/8 in. deep, is cut with its inner edge 5/8 in. from the outer edge of F' and F ; this serves as a collecting reservoir for the crushed material. The pistons are driven by a Denbigh No. 4

fly-press (manufactured by Thomas Ward, Ltd., Sheffield) worked by hand. The block stands on an 8-in. square by 1/2-in. mild steel plate when being used.

Method of manufacture.—Each side (A and B) of the block was cut from a 4-in. by 2-in. billet of steel and trued square in a shaping machine. Care was taken to remove the smallest possible amount of steel at this stage. The two sides were clamped together, and 3/8-in. holes (M and M′) drilled in the position shown in Fig. 1 for the locating pins. These holes (M and M′) were widened to 1/4 in. on the inner side. The inner surface of each side was ground on a surface grinder and hand-lapped with fine grinding paste on a flat steel plate. They were then clamped together with the placing pins in position and the external surfaces milled to give a final finish, the sharp edges chamfered and 5/8-in. channels for the bolts machined in the ends. The bolts were then placed in position, tightened, and the cylindrical holes (D and E) drilled. For this, two sets, one 7/16 in. and the other 11/16 in., drill and reamer were used. The block was then dismantled, the reservoir channel (K) cut in one face with an end miller and the internal surface of each side of the block was again lapped with grinding paste. Finally the sharp edge of each of the holes was then filed back about 1/16 in. as indicated by a thickened line in Fig. 1. The angle of the filed surface is such that when the blocks are clamped together a V-shaped joint is formed. The pistons (F and F′) were turned in a lathe from 1.1/8 in. stainless rod and fitted to each hole with a tolerance of 2/1000 to 3/1000 in. The adaptor (H) for the fly-press was turned from 2-in. stainless bar and a 5/16-in. Whitworth grub screw added to secure the plungers. The press may be obtained from Shandon Scientific Co., Ltd., 6, Cromwell Place, London, S.W.7.

Care of the press.—Even though the press is made of stainless steel, the ground surfaces quickly discolour if left wet. To prevent this the press is cleaned immediately after use, under running hot water, with the aid of a soft test-tube brush and a neutral detergent. After a thorough rinsing in hot water the separate pieces (whilst still hot) are flooded with methylated spirits. When dry, silicon grease is applied and wiped off again. The press is kept assembled in order to avoid the accumulation of grit between the ground surfaces. The plungers are cleaned and treated in the same way. On the first few occasions when abrasive was used the surface exposed to the passage of the material became polished. Thereafter wear was extremely slight. Care must be taken to avoid scratching the interface and plungers with large glass particles, such as might occur from breakage of centrifuge tubes or glass rods during the preparation of bacterial suspensions. On one occasion when this happened it was found necessary to mill the surfaces flat and grind and lap them together again. The holes were then re-bored and reamered. For this purpose the set of drills and reamers has been set apart and is not used in any other work. Damage to the plunger and to the hole of one press resulted from the plunger being forced in at an angle. The surfaces were reground to reduce the size of the holes, which were re-bored and reamered ; a new plunger was also made in this case.

Abrasives.

Powdered Pyrex glass was prepared in a ball mill made available by the courtesy of the Department of Glass Technology of this University. Broken pyrex glass was cleaned with chromic sulphuric acid mixture, washed, dried and

loaded (8 lb.) into a cylindrical steel ball mill (18 in. diam., 30 in. long) containing 3-in. stainless steel balls. Grinding was carried out over a period of 9½ hours until the average section of the particles was 5·0μ^2 (range 1·0 to 150μ^2). Further grinding for three hours did not reduce the size of the particles but rounded their edges. A portion of 200 g. of powdered glass was heated with 2 N HCl and washed with water until the washings gave no test for chloride. The washed and unwashed powdered glass gave identical results when used to prepare glutamic acid decarboxylase and formic hydrogenlyase. Triple-washed emery powder was obtained from Messrs. G. Cannings, Ltd., Sheffield. Samples of silica and zircon dusts were obtained as gifts through the courtesy of Messrs. Colin Stewart, Winsford, Cheshire. Polishing alumina (Griffin & Tatlock) was washed and dried as described by McIlwain (1948), but a cheaper alumina (Metallurgists' aluminum oxide) and an alumina marketed as " Diamantine " were obtained from Messrs. J. Preston, Ltd., Sheffield, as dry powders and were not washed.

Chemical and bio-chemical determinations.

Adenosine triphosphate (ATP) was prepared by the method described by Umbreit, Burries and Stauffer (1949), and was 98 per cent pure as judged by spectrophotometric estimation of the adenine and total and labile phosphorus. Adenosine diphosphate (ADP) adenosine and adenine were commercial specimens. L-glutamine and L-glutamic acid were the specimens used previously (Hughes, 1950).

Glucose was determined by the method of Somogyi (1945) and Nelson (1944); lactic acid by the method of Friedmann and Graeser (1933). Ammonia was determined by Nesslerization after distillation in a modified Parnas apparatus. Phosphorus was determined by the method of Lowry and Lopez (1946).

Manometric experiments were carried out in the Warburg apparatus.

Glutamic acid decarboxylase was determined manometrically by a modification of the method of Gale (1947): each Warburg flask contained 1·0 ml. 0·05 M-acetate buffer, pH 4·5, + 0·5 ml. 0·1 M-glutamate, in the main compartment and 0·5 ml. enzyme preparation in the side bulb. Yellow phosphorus was in the centre well. The gas phase was N_2 and the temperature 30°.

Fermentation was followed by measuring the formation of acid as judged by the evolution of CO_2 from a bicarbonate buffer. Medium (1·0 ml.) consisting of 2·0 ml. 0·05 M hexosediphosphate, 2·0 ml. 0·02 M-ATP, 2·0 ml. boiled yeast extract, salts " B " (Barton-Wright, 1946) 0·05 ml. in 10 ml. of water was added to the main compartment of a Warburg cup together with 0·5 ml. 0·1 M-$NaHCO_3$, and 0·5 ml. enzyme preparation. In the side bulb was 0·5 ml. 0·1 M-glucose. Yellow phosphorus was in the centre well. The gas phase was N_2/5 per cent CO_2 and the temperature 40°. In some experiments $NaHCO_3$ was replaced by 0·1 M-phosphate buffer, pH 7·8, and the gas was replaced by N_2.

Formic hydrogenlyase activity was measured by following H_2 production from formate as follows: each Warburg cup contained 0·5 ml. enzyme preparation and 0·5 ml. 0·04 M-phosphate buffer, pH 6·2, in the main compartment and 0·2 ml. 0·1 M-formate in the side bulb. Paper and KOH were in the centre well. The gas phase was H_2 and the temperature 30°.

Fumarase activity was followed manometrically by measuring CO_2 evolution in the presence of malate-adapted cells of *Lactobacillus arabinosus* (Korkes and Ochoa, 1948) under the conditions described by Nossal (1951).

Growth and collection of organisms.

Cl. welchii, N.C.T.C., Strain SR 12 was grown for 3 to 16 hr. on the meat medium described by Krebs (1948). *L. arabinosus* 17–5, *Leuconostoc mesenteroides* P 60, *Streptococcus faecalis* N.C.T.C. 6782 were grown for 16 hr. at 37° on the following medium : Pronutrin (a commercial casein hydrolysate marketed by Herts Pharmaceuticals Ltd., Welwyn), 20 g. ; glucose, 20 g. ; hydrated Na-acetate, 10 g. ; KH_2PO_4, 4·5 g. ; N-NaOH, 26 ml. ; boiled yeast extract (Krebs, 1948), 40 ml. ; were dissolved in tap water, the pH adjusted to 7·5 and the volume made up to 1000 ml. The medium was sterilized by autoclaving for 10 min. at 120°. *Proteus morgani* N.C.T.C. 2010 and several strains of *Bact. coli* were grown for 12 hr. at 37° on a medium consisting of 2 per cent bactopeptone, 0·1 per cent yeast extract, 2 per cent glucose. *Mycobacterium phlei* N.C.T.C. 625 and *Mycobacterium smegmatis* N.C.T.C. 623 were grown for 4 days at 37° on infusion broth containing 0·5 per cent glycerol. *Neurospora crassa* (Imperial Mycological Institute, Strain 3411) was grown on a modified Czapek-Dox medium for 14 days at 30°. Bakers' yeast was obtained commercially as marketed by Distillers' Company, Ltd. Pressed brewers' yeast was obtained from a local brewery. After growth, all cells with the exception of *Neurospora* were collected by centrifugation, washed with 0·9 per cent NaCl and centrifuged again. The *Neurospora* was collected on a small Buchner funnel, the solid mat dried between filter-paper, homogenized for 5 min. with five times its own weight of water in a Waring blendor and the broken-up mat collected by centrifugation.

Crushing operation.

To prepare the press for crushing, the inner surfaces of the blocks were cleaned and lubricated with a silicon lubricant (stopcock grease, Dow Corning Corporation, Midland, Mich., U.S.A.), which was then wiped off cleanly with a cloth. The surfaces were then lightly rubbed with clean fingertips in order to remove traces of grit, the press assembled, clamped together by the bolts and cooled. The temperature to which the block was cooled depended on the type of material to be crushed. When crushing was to be carried out with the aid of abrasives the block was cooled to + 2° to − 15° ; for crushing without abrasive it was cooled to − 20° to − 35° on solid CO_2. When thoroughly cooled, the material to be crushed was transferred into one of the holes and the plunger (not previously cooled) pushed in by hand. The bolts were then tightened, and the plunger pushed down by the press until the material was packed into a solid block. Without clamping the plunger with the clamping bolt on the slide of the fly-press the handle was turned about half to three-quarter of a revolution to raise the slide about 1 to 1.1/2 in. and was then swung down as rapidly as possible, raised and swung down again until the material was forced across the interface and into the reservoir. It was found by measurements with the aid of a Brunnel hardness tester that force of the blow was in the order of 12–15 tons per square inch. The greatest pressure was exerted when the handle of the " fly-press " was held rather than when it was merely given a single swing and allowed to descend under its own momentum. Under these conditions crushing with abrasive took 0·5–1·0 seconds, and one or three blows depending upon the temperature of the block and the amount and nature of the material. When bacteria were crushed without the addition of abrasive the block was cooled to below − 20°,

and the number of blows needed to force the material into the reservoir varied with the wet weight of the cells. With 0·5 to 2·0 g. in the smaller hole usually one blow was sufficient. With 2·0 to 8·0 g. of cells in the larger hole it was usually found necessary to give up to six blows to the plunger. Sometimes not all of the material was forced into the reservoir, but that which remained in the hole was generally found to be as efficiently crushed as that in the reservoir.

The block was removed from the fly-press after crushing, the bolts loosened, tapped out of the channels and the two sides levered apart with the aid of the plunger. The crushed material in the reservoir channel was generally in the form of a frozen block. Subsequent treatment depended on the enzyme system being studied, and some examples are given in later sections.

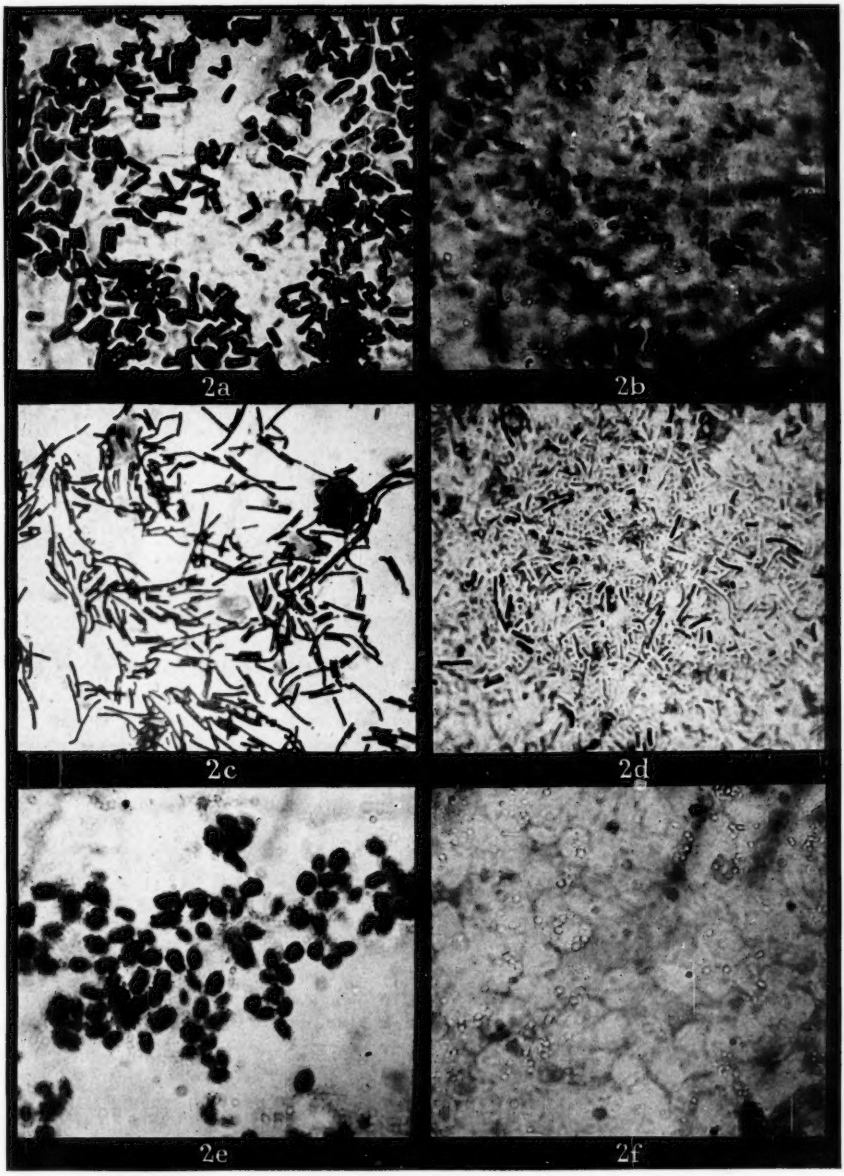
Estimation of cell disintegration.

Where crushing was carried out with the addition of abrasives, the number of cells which remained after crushing was estimated by examination of fixed stained smears mounted either in immersoil or in water as described by McIlwain (1948). The stains used depended on the properties of the organism ; where the cell was Gram-positive a full Gram stain was used ; acid-fast organisms and spores were stained with carbol-fuchsin, decolourized and counter-stained with malachite green. Gram-negative cells were stained with Loeffler's methylene blue or dilute carbol-fuchsin, and as these cells were difficult to detect when mixed with abrasive and cell debris it was usual for at least two people to examine the slides. Where crushing was carried out without the addition of abrasive the number of cells remaining was estimated by suspending a known weight (0·05 to 0·1 g.) of material in buffer and diluting in 0·9 per cent NaCl tinted with methyl violet. The intact bacteria in the suspension before and after crushing were counted in a Thoma haemocytometer. Material which was difficult to suspend, e.g., *Myco. phlei* before crushing and *Cl. welchii* after crushing, was suspended with the aid of a Potter type stainless steel homogenizer. In some of the later experiments cells were counted by mixing the bacterial suspension with horse blood as follows : 2·0 ml. of a suspension of the material (0·1–0·2 g./2·0 ml.) was mixed intimately with 1·0 ml. of blood, and smears of the mixture stained with Leishman's stain. Intact bacterial cells and erythrocytes were counted with the aid of a 1/12 in. oil-immersion lens and a 4 mm. square diaphragm in the eye-piece ; about 20 fields or about 1000 erythrocytes were counted in each smear. Not all species of crushed bacteria could be counted in this way since some, e.g., *Cl. welchii*, after crushing appeared to cause lysis of the erythrocytes. Where

EXPLANATION OF PLATE.

FIG. 2.—Microscopic appearance of stained smears of micro-organisms before and after treatment in the press (× 980).

a. *Cl. welchii* before crushing. Gram's stain.
b. *Cl. welchii* after crushing. Gram's stain. Showing Gram-negative cytoskeleton on a dense Gram-negative background.
c. *L. arabinosus* before crushing. Gram's stain.
d. *L. arabinosus* after crushing. Gram's stain. Showing some apparently undamaged cells, many Gram-negative cytoskeletons and some increase in background staining.
e. Bakers' yeast before crushing. Leishman's stain.
f. Bakers' yeast after crushing. Leishman's stain. Showing many empty and partially emptied cells and increased background stain.


2a
2b
2c
2d
2e
2f

no lysis occurred this method agreed well with those used in earlier experiments. Viable cells have not been counted, since the main object was to extract enzymes from the crushed material and complete sterilization was not therefore essential. Where the term " complete " or " 100 per cent disintegration " is used it indicates that no whole cells could be found by examination of at least two separate samples by any of the above methods.

RESULTS.

Results of crushing various micro-organisms.

A number of species of micro-organisms frequently used in enzymic studies were crushed in the press by the above method. In most cases distintegration was complete when abrasive was used, and in many cases 80–90 per cent disintegration occurred when abrasive was omitted (Table I). In addition to crushing organisms which are known to be relatively easily disintegrated, e.g., *Cl. welchii*, it will be seen that the press crushed organisms such as yeast and *Mycobacteria*, which are difficult to disintegrate by previous available methods (cf. Edson and Hunter, 1947). This result was noteworthy, since the optimum conditions for complete crushing were established by experiment only in the case of *Cl. welchii* and *Bact. coli*. It seems likely, therefore, that the proportion of abrasive to cells is much less critical in the present method than is the case with hand and mechanical grinding (McIlwain, 1948 ; Dockstader and Halverson, 1950).

Properties of stained smears.

Crushed Gram-positive organisms lost their ability to retain the Gram stain, and smears were stained uniformly Gram-negative. In the absence of abrasive, some, e.g., *Cl. welchii*, *L. arabinosus* and *Bacillus subtilis*, left Gram-negative cytoskeletons (Table I, and Fig. 2 a and b, 2 c and d). In all micro-organisms tested disintegration of the cell upon crushing was indicated by a great increase in background staining. This was well demonstrated by adapting a method recently used to develop protein spots on paper (Durrum, 1950). Smears were fixed at 80–100° for 5 min., and the slide was immersed for 5 min. in a saturated ethanolic solution of mercuric chloride containing 0·5 per cent bromthymol blue, washed 10 min. in running tap-water and dried by blotting. Intact cells appeared lightly stained green-blue, damaged cells were generally only partially stained, and the escaped cell contents appeared as a heavily blue-stained background. Smears of crushed *Mycobacteria* examined by this method showed that a high proportion of cells which retained their acid-fast properties after crushing had probably lost some of their cell contents. Leishman's stain was particularly valuable for examining smears of crushed yeast (Fig. 2) ; about 50 per cent of yeast cells which retained Gram's stain after crushing were found to have lost the whole of their cell contents. In making counts these damaged cells were included as whole cells and estimations of disintegration in Table I therefore tend to be low. In the case of yeast, for instance, if damaged cells are excluded the percentage disintegration would be 75 instead of 50 with abrasive and 90–95 instead of 80 without abrasive. A high proportion of the spores of *B. subtilis* left after crushing the vegetative cells were found to stain with malachite green instead of with carbol-fuchsin in the normal manner.

TABLE I.—*Results of Crushing Micro-organisms in the Press.*

(Details of the operation are as described fully in the text.)

Organism.	Part of powdered glass added to 1 part of wet wt. of cells.	Temperature of press (° C.).	Degree of cell disintegration (per cent).	Microscopical appearance of stained smears after crushing.
Cl. welchii	3·0	+ 2	80	When disintegration was complete the background was stained uniformly Gram-negative. When disintegration was incomplete damaged and undamaged Gram-positive cells were superimposed on a Gram-negative background.
	2·0	+ 2	100	
	1·0	+ 2	60	
	1·0	− 10	100	
	0·8	− 20	100	
	0·8	− 35	80	
	None	− 35	80–90	Damaged and undamaged cells were superimposed on a red field consisting of a faint Gram-negative background and masses of Gram-negative cytoskeletons (Fig. 2 a and b).
L. arabinosus	1	− 15	100	Uniform Gram-negative background.
	None	− 35	80	Gram-negative background with superimposed Gram-positive cells. Some cytoskeletons (Fig. 2 c and d).
Bact. coli	1·0	− 10	100	No discrete cells and general Gram-negative background staining.
	0·8	− 20	100	As above.
	None	− 35	90–95	Discrete and apparently undamaged cells superimposed on a Gram-negative background.
Proteus morgani	1·0	− 20	100	As in *Bact. coli*.
	None	− 35	80–90	—
Strep. faecalis	1·0	− 15	100	No Gram-positive cells and general Gram-negative background staining.
	None	− 35	80	Undamaged Gram-positive cells superimposed on Gram-negative background. No cytoskeletons.
Leuc. mesenteroides	1·0	− 15	100	As *Strep. faecalis*.
Myco. phlei	1·0	− 10	100	No discrete acid-fast organisms and general background staining.
	None	− 35	*c.* 80	Very many cells had lost portions of the acid-fast material and appeared to have holes or pieces chopped out of them. The background staining was fairly uniform.
Myco. smegmatis	1·0	− 10	*c.* 90	Some cells were in clumps and appeared to be undamaged, probably due to the initial difficulty of mixing the cells uniformly with the abrasive.
	None	− 35	*c.* 60–80	Deep background staining upon which damaged and undamaged cells were superimposed in clumps.
Neurospora crassa	1·0	− 10	No estimation possible	Background staining increased, and there was an almost complete disappearance of discrete mycelia.
Bakers' yeast	1·0	− 15	50	When crushed both with or without abrasive many Gram-positive cells were seen superimposed on a deeply stained Gram-negative background. Smears stained with Leishman showed that many cells which appeared undamaged when stained with Gram were empty (Fig. 2 f and g).
	None	− 35	80	
B. subtilis	1·0	− 15	100	All vegetative cells disappeared and films were Gram-negative with a few cytoskeletons. Many spores remained but had changed their staining reactions.

The use of abrasives other than powdered glass.

Since abrasives vary widely in their adsorptive properties it is of advantage in the preparation of any enzyme from a micro-organism to have available as many abrasives as possible. To test abrasives they were mixed with washed cells (4 g. wet wt.) of *Bact. coli* and *Cl. welchii* in successive 0·2 g. portions until the mixture formed a fairly stiff paste. This was added to the largest hole of the press at — 10° and crushed as described. In some cases disruption was complete. In others disruption was less complete (Table II), probably because too much abrasive

TABLE II.—*Cell Disintegration with Abrasives other than Powdered Pyrex Glass.*

Organism.	Abrasive.	Parts of abrasive added to 1 part of wet weight of cells.	Amount of cell disintegration (per cent).
Bact. coli	Alumina	2·0	100
	Diamantine	1·25	90
	*Aluminium oxide	1·1	50
	Zircon (200 mesh)	2·8	100
	,, (flour)	2·3	95–100
	Triple washed emery	3·0	80
Cl. welchii	Alumina	1·0	100
	Carborundum flour	2·0	100
	Silica (flour)	2·5	90
	Zircon (,,)	1·0	100

* This material was very adsorbing and the mixture became dry and very hard as it was transferred to the press.

had been added to the cells. Since these experiments were performed, Dockstader and Halverson (1950) have reported that alundum or Super-Cel may be used for grinding bacteria. It is likely that these may also be used in the press. Subsequent work showed that the proportion of abrasive to cells must be found by experiment with each organism and with each abrasive.

Amount of material used in the press.—When abrasive was used a total wet weight of 1·0 to 4·0 g. of mixture was crushed in the smaller hole and from 4·0 to 16·0 g. in the larger hole. This means that the amount of wet cells that can be crushed with abrasive in the press is from 0·5 to 8·0 g. In a larger press, with a single hole of 7/8 in. diam., 12·0 g. wet weight of *Cl. welchii* mixed with 10 g. of powdered glass was crushed with 90 per cent cell disintegration. When abrasive was omitted 0·8 to 4·0 g. wet weight of cells were crushed in the smaller and 4·0 to 6·0 g. in the larger hole.

Preparation of enzymes from micro-organisms crushed in the press.

Much of the work presented in this section forms part of more extended studies, fuller details of which will be published elsewhere. I am particularly grateful to Miss F. Gilchrist, Microbiology Department, Sheffield, for permission to include some results of her studies of formic hydrogenlyase.

Enzymes from Cl. welchii.—Extracts containing glutamic acid decarboxylase may be prepared from *Cl. welchii* by grinding the cells by hand with powdered glass and extracting with buffers (Hughes, 1949). Extracts of equal or slightly higher activity were prepared from cells crushed with powdered glass in the press (Exp. B and C, Table III). About 90 per cent of the activity was extracted when

TABLE III.—*Glutamic Decarboxylase Activity of Various Preparations from* Cl. welchii.

Enzyme preparation.	CO_2 evolved (μl./mg. dry wt. cells or extract from cells/30 min.)
A. Intact washed cells	246
B. Extract from cells broken by hand-grinding with powdered glass	240
C. Extract from cells broken in the press with powdered glass	248
D. ,, ,, ,, ,, with silica	5
E. ,, ,, ,, ,, without abrasive	220
F. ,, of cells frozen on solid CO_2 and thawed five times successively	105

crushing was carried out without abrasive (Exp. E, Table III) and at — 20°; and about 30 per cent Gram-positive cells remained but many of these appeared to be damaged. Material from *Cl. welchii* which had been crushed in the absence of abrasive was extremely mucoid, and could only be evenly suspended with the aid of a Potter type homogenizer or Waring Blendor. About 40 per cent of the enzyme activity was extracted from cells which had been frozen at 40° and thawed five times successively (Exp. F, Table III); this material was not mucoid and almost all the cells stained Gram-positive. The crushing operation is therefore necessary for high yields of enzyme activity in the absence of abrasive. All extracts except those crushed with silica (Exp. D, Table III) contained glutaminase as well as the decarboxylase as judged by ammonia production from glutamine. The glutaminase and not the decarboxylase could be extracted from material crushed with silica.

Extracts which fermented a mixture of glucose and hexose diphosphate at approximately the same rate as intact cells (10μ mol./mg. dry wt./hr.) were obtained from young (3–4 hr. growth) *Cl. welchii* which had been crushed at — 35° in the absence of abrasive. The extracts were prepared by homogenizing the crushed material (7·0 g. wet wt.) in 12·0 ml. of 0·05 M $NaHCO_3$ containing 1·2 mg. cysteine in a cold Potter type homogenizer and adjusting the pH to 8·0 with N-NaOH. The homogenate was centrifuged for 10 min. at approx. 8000 r.p.m. in a Sorval high-speed centrifuge previously cooled to — 5°. The opalescent supernatant contained 50 per cent of the dry weight of the cells. Fermentation during the first 20–40 min. produced acid, CO_2 and a gas assumed to be H_2, in the molar proportion of 2 : 1 : 1. Thereafter acid and H_2 production fell progressively, but CO_2 production continued at almost its initial rate. Only small quantities of lactic acid appeared in the fermentation medium, and the acid produced has not been identified. Cells dried from the frozen state and cells which had been frozen and thawed produced acid at approximately 20 per cent of the rate of the above extracts, but did not produce CO_2 and H_2 under these conditions. Extracts of crushed cells also produced inorganic P from 0·05 M ATP at the rate of 1·0–3·0 mμ. mol./extract from 1·0 mg. dry wt. cells/hr.

Enzymes from L. arabinosus.—Extracts of *L. arabinosus* prepared as described for *Cl. welchii* formed lactic acid from a mixture of glucose and hexosediphosphate at the rate of 0·1–0·2μ mol./mg. dry wt. cells/hr., i.e., about 1/10 to 1/20 the rate in intact cells. Glucose alone was not fermented. Similar results were given

by extracts of cells crushed with the aid of either pyrex glass or polishing alumina. The extracts deaminated nicotinamide at the same rate as intact cells (2μ mol. NH_3 formed/mg. dry wt./hr.)—cf. Hughes and Williamson (1950). Neither ATP, ADP, adenosine nor adenine were deaminated under a variety of conditions. Inorganic phosphate was produced from ATP by the extracts (0·03μ mol./mg. dry wt. cells/hr.) and ADP (0·01μ mol./mg. dry wt. cells/hr.), but not from adenylic acid.

Enzymes from Strep. faecalis.—Extracts prepared from crushed *Strep. faecalis* as described for *Cl. welchii* formed lactic acid from glucose in the absence of hexosediphosphate at the rate of 0·5 to 2·0μ mol./mg. dry wt./hr., i.e., about 1/5 to 1/10 the rate of intact cells. The addition of ATP (0·005 M) to the medium increased the rate of CO_2 production in a bicarbonate medium, but did not increase the amount of lactic acid formed. It is assumed that this is due to the formation of inorganic phosphate from ATP by phosphatase.

Enzymes from Bact. coli.—Extracts with formic hydrogenlyase activity were obtained from *Bact. coli* by crushing the cells with powdered glass in the press and extracting the ground mass with water (Table IV). Similar preparations

TABLE IV.—*Formic Hydrogenlyase from* Bact. coli.

Abrasive used to crush the cells.	Hydrogen production (μl./30 min.).
Polishing alumina	230
Diamantine	None
Zircon flour	138
Zircon (200 mesh)	302
Pyrex glass	281
Emery flour	350

were obtained from *Proteus morgani*. Comparison of hydrogenlyase activity in extracts prepared from cells crushed with a variety of abrasives showed that enzyme activity did not depend entirely upon the degree of cell disintegration, but depended also upon the abrasive used. For instance, extracts of cells crushed with diamantine, where cell disruption was complete, contained little enzyme activity, while extracts prepared from cells crushed with Zircon (200 mesh), where crushing was less complete, had high enzyme activity (compare Tables II and IV).

Enzymes from bakers' yeast.—Extracts of bakers' yeast were prepared by crushing the yeast cake at — 20° without the addition of abrasive and extracting the crushed material with an equal quantity of water. The opalescent extract showed no endogenous fermentation at pH 5·0 in the presence of fluoride (0·01 M). Its fumarase activity was from 7–9 times greater than that of the intact cells. The extract was thus a convenient source of fumarase, since the enzyme activity remained stable for at least 14 days when stored at — 15°. Similar preparations were made from brewers' yeast.

DISCUSSION.

Disruption of micro-organisms by abrasives.

The disruption of yeast (Buchner and Hahn, 1903) and bacteria (Wiggert *et al.*, 1940; Kalnitsky *et al.*, 1945) by grinding with abrasive is generally assumed to be due to rupture of the cell walls by shearing forces and by penetration of the

sharp abrasive particles (cf. Dockstader and Halverson, 1950). It appeared feasible therefore that rapid compression, leading to close packing of a mixture of abrasive and bacteria, would disrupt the cells more efficiently than grinding by hand. Preliminary experiments were made with a press consisting of a piston working in a seamless steel tube, plugged at one end with a removable steel plug. These showed that high pressure (>2000 lb./sq. in) applied slowly did not disintegrate bacteria, but that when the mixture was given a sharp and heavy blow in a mechanic's " fly-press " most of the cells were broken. In this earlier press both the plug and piston often became jammed when used at room temperature and at lower temperatures the mixture escaped. In order to avoid these faults the cylinder was split and the channel cut in the interface. Compared with the older methods of using abrasives much more consistent results are obtained with the press, e.g., more than 200 uniform batches of formic hydrogenlyase have been prepared from *Bact. coli*. Attention has already been drawn to other main advantages of the press.

Disruption of micro-organisms without the addition of abrasives.

Previous instruments for disrupting micro-organisms without the addition of abrasive belong to two main categories. The first includes the steel ball mill of Booth and Green (1938) and the glass mill of Muys (1949), where disintegration is brought about mainly by shearing forces. In the second group disruption is brought about largely by cavitation, set up by shaking with glass beads (Curran and Evans, 1942), or by vibrating with supersonic waves (Stumpf, Green and Smith, 1948). A press recently described by Milner *et al.* (1950) in which a bacterial suspension is forced through a fine nozzle also belongs in this category. These methods have the common disadvantage that the time taken for a high degree of disintegration is from 20 to 180 minutes for 1 to 3 g. of bacteria. It is also difficult to keep the crushed material cold and to crush certain types of bacteria, e.g., capsulated cells and *Mycobacteria*. The press described in the present paper when used at − 20° to − 35° and without the addition of abrasive therefore compares favourably with these former methods, since 80–90 per cent cell disintegration of a variety of micro-organisms was obtained in less than 5 sec. while the material was kept cold. It seems likely that disruption without the addition of abrasive in the press is due to ice crystals which act as abrasive (Weisser and Ostered, 1945). Re-gelation and the passage of the material across the interface may also assist in disintegration.

While this work was in progress Fraser (1951) described the bursting of *Bact. coli* in suspensions, by sudden release of gas pressure. In this method both the age of the cells and the concentration of the suspension appear to be critical. The age of the cells crushed in the press was not found to be critical, but a more direct comparison between the press and the method of bursting cells is not possible since Fraser reports results only with one organism.

Additional uses for the press.

Examination of stained smears of bacteria crushed in the press suggests that the press might be used for cytological studies. It is likely that endotoxins and other labile material from inside micro-organisms may also be prepared from material crushed in the press.

SUMMARY.

1. Details are given of a simple press consisting essentially of a piston moving rapidly in a cylinder and in which various micro-organisms were disintegrated. Abrasives of various types were added to the cells between + 2° and — 20° ; no abrasive was added between — 20° and — 35° because ice crystals can act as abrasive.

2. The main advantages of the press over other available methods for disrupting micro-organisms are that the time taken is short (0·5 to 5·0 sec.), the material is kept cold (+ 2° to — 35°), and the cells are not diluted in suspending fluid.

3. The microscopic appearance of cells after crushing is described, and details are given of the preparation of some bacterial enzymes from crushed material.

Especial thanks are due to Mr. G. Fletcher, who made the press, and without whose skill this work would not have been possible. The author is grateful to Prof. H. A. Krebs, F.R.S., for helpful advice and criticism, and to Miss F. Gilchrist and Mr. P. M. Nossal for permission to publish details of some of their experiments. Thanks are due to Messrs. Colin-Stewart for gifts of abrasive.

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THE GROWTH OF INFLUENZA VIRUSES AND NEWCASTLE DISEASE VIRUS IN MOUSE BRAIN.

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VARIOUS strains of influenza virus have been adapted to kill mice after intracerebral inoculation in high dilution (Stuart-Harris, 1939; Francis and Moore, 1940). Without a preliminary period of adaptation they are capable of interfering with the growth and lethality of some neurotropic viruses (Vilches and Hirst, 1947), and also, when inoculated with a neurotropic strain of influenza, of acquiring neurotropism (Burnet and Lind, 1950, 1951).

There have been several reports that influenza virus does not multiply appreciably on first introduction into a mouse's brain. Thus Henle and Henle (1944, 1946) and Vilches and Hirst (1947) demonstrated a steady decline of the virus titre in the brain during the days following intracerebral inoculation. However, with the finding that about 95 per cent of any intracerebral inoculum passes into the circulation (Cairns, 1950), it became obvious that much of the reported fall in titre was due to this unavoidable wastage, since the drop in titre had always been measured from a calculated initial titre, allowing for no wastage, rather than from any observed initial titre.

This paper reports the results of a re-investigation of the behaviour of various unadapted strains, and a comparison of their behaviour with that of an adapted neurotropic strain. The findings conflict with those of Schlesinger (1950), who reported that unadapted strains, although producing haemagglutinin, show no detectable multiplication of infectious virus.

MATERIALS AND METHODS.

Virus strains.

Influenza A: WS (Smith, Andrewes and Laidlaw, 1933).

Neuro-WS (Stuart-Harris, 1939).
PR8 (Francis, 1934).
MEL (Burnet, 1935).

Influenza B: LEE (Francis, 1940).

Swine influenza: (Shope, 1931).

Newcastle disease virus, NDV. Victorian strain (Albiston and Gorrie, 1942).

All these strains were used in the form of allantoic fluids from eggs infected on the 11th day of incubation and harvested after a further 48 hours' incubation; all such fluids were stored in capillary tubes or ampoules, which were kept in a dry ice cabinet and removed from there only immediately before use.

Unfortunately a detailed history of these strains, since isolation, is not available; all had been subjected to repeated allantoic passage at dilutions of 10^{-4} to 10^{-6} before their use in these experiments; with the exception of NDV and neuro-WS, all had, at some stage in their laboratory career, been adapted to kill mice on intranasal inoculation.

Horse serum saline.—All dilutions of materials for inoculation were made with 10 per cent inactivated horse serum in normal saline.

Intracerebral inoculation.—Inoculation of 0·03 ml. was made through a 25-gauge needle into the right hemisphere. For this the mice were lightly anaesthetized with ether and chloroform. The mice came from the Institute animal rooms and were used when 4–5 weeks old, the sexes being represented randomly.

Titration of virus infectivity.—3·16-fold (0·5 $\log_{10}$) dilutions of the test material were inoculated sub-allantoically into groups of two or three 10–12-day eggs. After 3 days' incubation at 35°C. each allantoic fluid was tested for the presence of haemagglutinin by adding 0·1 ml. of the fluid to 0·25 ml. 1 per cent fowl red cells, haemagglutination being assessed after half an hour at room temperature (or at 4°C. in the case of NDV). Titres were calculated by the method of Reed and Muench (1938).

The infectivity titre of virus in mouse brain was obtained from a pool of 4 or 8 brains. The mice were killed by chloroform, and each brain was suspended in 1·5 ml. of horse serum saline after grinding with alundum (this is roughly equivalent to a 15 per cent suspension, v/v). The titres were finally expressed per brain. In every case the eggs were inoculated within half an hour from the time the mice were killed.

EXPERIMENTAL.

The growth of neuro-WS virus.

The strain of neuro-WS used in this laboratory was derived from the original variant isolated by Stuart-Harris (1939), and kills adult mice 3–9 days after intracerebral inoculation. Comparison of its titre in mice (LD50) with its titre in the allantoic cavity (ID50) shows that, when allowance is made for the inefficiency of titration in mice (95 per cent wastage of the inoculum, or a reduction of any titre by 1·3 $\log_{10}$ units), the virus is about one hundred times as infectious for mice as for the allantoic cavity, whether the virus is used in the form of mouse brain suspension or allantoic fluid; similarly the virus, in the form of infected allantoic fluid, is about one hundred times as infectious for the amniotic cavity as for the allantoic cavity (Table I).

TABLE I.—*Infectivity of Neuro-WS in the Form of Brain Suspension and Allantoic Fluid, for Mice and for the Allantoic and Amniotic Cavities.*

Source of virus.	Allantoic cavity ID50.	Mice (intracerebral).		Amniotic cavity ID50.
		LD50.	Corrected LD50.*	
Brain	$10^{3.7}$	$10^{4.3}$	$10^{5.6}$	—
Allantoic fluid	$10^{5.2}$	$10^{5.5}$	$10^{6.8}$	$10^{7.1}$

* 1·3 added to the $\log_{10}$ titre, to compensate for 95 per cent wastage.

Titration of infectivity in the allantoic cavity: 5 eggs per 0·5 $\log_{10}$ dilution. Titration of infectivity in mice: 6 or 8 mice per 0·5 $\log_{10}$ dilution. Titration of infectivity in the amniotic cavity: 7 eggs per 1·0 $\log_{10}$ dilution.

Thus neuro-WS appears to be exceptional among described strains of influenza virus, since it exists in a stable state of unadaptedness to the allantoic cavity despite eight successive allantoic passages at high dilution before use in these experiments. Nevertheless, for the sake of simplicity it was decided to measure the growth rate of neuro-WS in mouse brain by titration in the allantoic cavity; while the maximum titre reached in the brain would be underestimated, the rate of growth might well be estimated correctly.

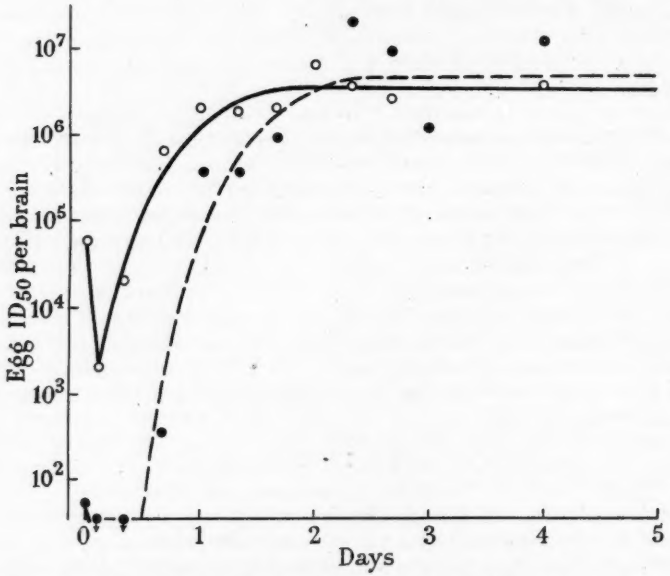

FIG. 1.—Growth of neuro-WS in mouse brain, after inoculating 10^4 (continuous line) and 10^1 (dotted line) mouse LD50. Each point represents a pool of 8 brains, titrated with 2 eggs per 0·5 $\log_{10}$ dilution. The values at 0 hours were calculated and do not allow for wastage of the inoculum.

Fig. 1 shows the growth of neuro-WS, after inoculation of 10^1 and 10^4 LD50 Growth is seen to be logarithmic up to a maximum titre of about $10^{6.5}$ allantoic —ID_{50} per brain. This maximum titre is the same for both inocula.

The growth of unadapted strains.

Allantoic fluids, representing various strains, were inoculated after dilution to 10^{-2} to $10^{-2.5}$. With such dilution the titre in the brain during the first few hours is considerably below the maximum titre reached by neuro-WS, and so saturation of the system cannot readily account for any failure to observe multiplication. Each strain was followed at 6-, 8-, or 12-hour intervals for a period of 2–4 days. Fig. 2 shows the results for MEL, WS, PR8, Swine, and LEE.

At least in the case of MEL definite increase in titre occurred somewhere between the 8th and 24th hour—a result confirmed on four separate occasions.

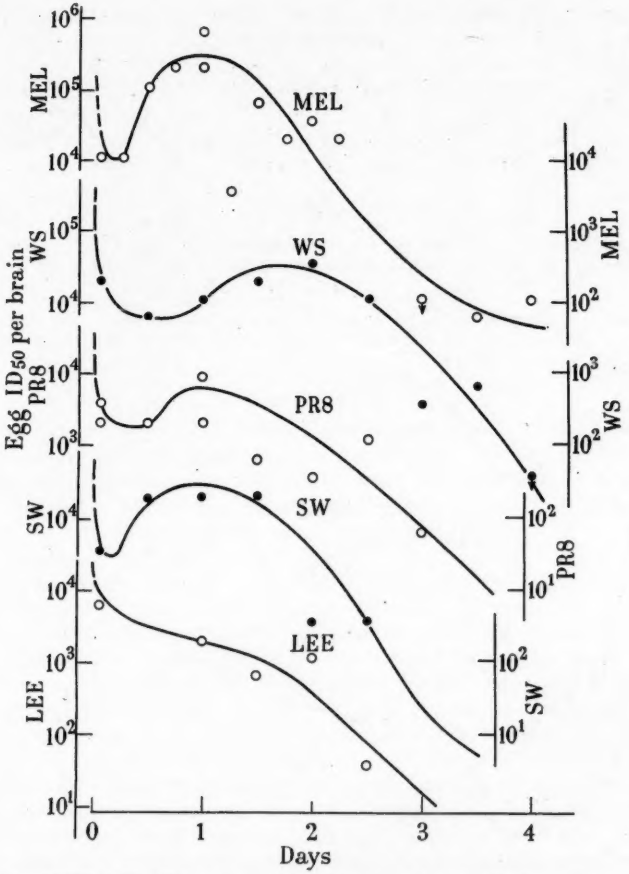

FIG. 2.—Growth of MEL, WS, PR8, swine and LEE in mouse brain. Each point represents a pool of 4 brains, titrated with 2 eggs per 0·5 $\log_{10}$ dilution.

With the exception of Lee the other strains at least maintained their titre from the 2nd hour to the 3rd day.

The behaviour of MEL was studied further, with hourly titration to locate the time at which increase in titre occurred. Later WS, PR8, NDV, neuro-WS and LEE were followed in a similar manner. All the strains except Lee showed a rise in titre which was transient save in the case of neuro-WS. The results of a typical experiment, using PR8, are given in detail in Table II, and the combined results for all the strains are shown in Fig. 3.

The time of the first rise and its transience varied for the different strains. With NDV a clear second rise was detectable. After this initial phase the unadapted strains showed a fairly steady titre, while that of neuro-WS continued to rise.

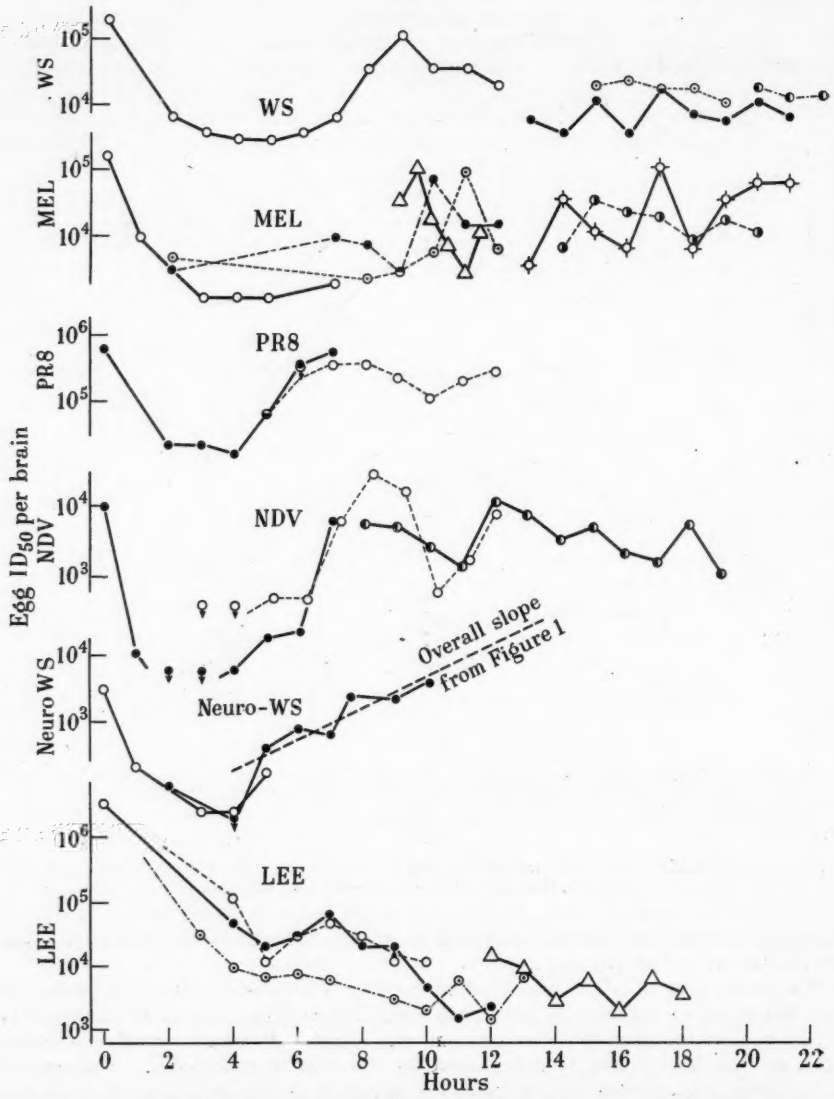

FIG. 3.—Growth of WS, MEL, PR8, NDV, neuro-WS and LEE in mouse brain. Each point represents a pool of 4 brains, titrated with 3 eggs per 0·5 $\log_{10}$ dilution. The results from groups of mice inoculated on different days are represented by different types of lines and points. The values at 0 hours were calculated and do not allow for wastage of the inoculum.

TABLE II.—*Egg-infectivity of Mouse Brains 2–7 hours after Inoculation with* $10^{5.1}$ *Egg-ID*50 *PR*8.

	$10^{-1.5}$.	$10^{-2.0}$.	$10^{-2.5}$.	$10^{-3.0}$.	$10^{-3.5}$.	$10^{-4.0}$.	$10^{-4.5}$.	$10^{-5.0}$.	Titre per 0·05 ml.	Titre per brain.*
2 hours†	3/3‡	2/3	1/3	2/3	1/3	0/3	0/3	0/3	$10^{2.75}$	$10^{4.33}$
3 ,,	3/3	3/3	2/3	1/3	0/3	0/3	0/3	0/3	$10^{2.75}$	$10^{4.33}$
4 ,,	3/3	3/3	2/3	0/3	0/3	0/3	0/3	0/3	$10^{2.60}$	$10^{4.18}$
5 ,,	3/3	2/2	3/3	2/3	1/3	0/3	0/3	0/3	$10^{3.25}$	$10^{4.83}$
6 ,,	3/3	3/3	3/3	2/3	2/3	3/3	0/3	0/3	$10^{4.00}$	$10^{5.58}$
7 ,,	3/3	3/3	3/3	3/3	2/3	2/3	2/3	0/3	$10^{4.20}$	$10^{5.78}$

* As each egg was inoculated with 0·05 ml., the titre per 0·05 ml. of brain suspension was converted to the titre per 1·9 ml. suspension (the total volume of brain and suspending fluid) by multiplying by 38.

† At each stated time four mice were taken.

‡ The enumerator represents the number of eggs showing the presence of haemagglutinin at 72 hours; the denominator, the number of eggs inoculated and alive at 72 hours.

DISCUSSION.

With the exception of LEE, all the unadapted strains studied in this paper showed a phase of multiplication which was transient. With one strain a second cycle of reproduction was demonstrated; with the others only a single cycle could be detected. Before dealing with the results in detail, it is necessary to review some of the factors determining the infectivity titre of a virus during its multiplication.

The time for completion of one cycle of multiplication ("total-cycle time") must be composed of the "cell-entry time," the "intra-cellular time," and a final period ("extra-cellular time") required for the virus to reach the stage of entry into fresh cells. Once the intracellular virus starts the process of multiplication it ceases to be infectious (Hoyle, 1948; Henle, 1949), and so the "resting level" of infectivity, between the time of inoculation and that of completion of the first cycle, represents non-multiplying virus rather than the virus which is going to provide the subsequent rise in titre.

If "average burst size"* represents the multiplication factor—virus produced, divided by the virus disappearing at the beginning of the cycle—then the overall growth of a virus is determined both by its "average burst size" and by an "efficiency fraction" representing that fraction of the detectable virus which is going to disappear (i.e., contribute to "average burst size"). Only if both these factors are constant will the growth of a virus be logarithmic. Where there is a maximum titre which is irrespective of the size of the inoculum, both factors are probably decreasing as the maximum titre is approached, the "average burst size" because of auto-interference, the "efficiency fraction" because of failure of the virus to find cells in which it can disappear. Until the distinction can be drawn, "average burst size" has to include virus disappearing into cells which are going to yield no burst and virus which disappears by mechanisms other than entry into cells, save where such processes can be estimated quantitatively (viz., the initial wastage associated with intracerebral inoculation).

The above points deal with the overall behaviour of a virus up to the time of appearance of antibody. The changes in infectivity titre during the first

* Use of the term "average burst size" is not meant to imply that bursting of the cells accompanies liberation of virus.

cycle must be considered separately. After inoculation, virus enters cells, begins to multiply, and at the same time becomes non-infectious, so that the " resting level " of infectious virus drops. At the completion of the first cycle of multiplication infectivity returns and the infectivity titre of the tissue rises, only to fall again as the process is repeated. The height of this rise, on a logarithmic scale, must be a function not only of " average burst size," but also of the efficiency in becoming non-infectious (as the latter increases, the " resting level " is lowered, making the rise, when it comes, relatively greater). Thus the fact that NDV shows a markedly greater rise than do any of the other viruses does not imply that NDV necessarily has the greatest " average burst size," since this virus also shows the greatest drop in infectivity after inoculation ; rather it suggests that NDV, at least initially, has a great capacity for becoming undetectable (i.e., high " efficiency fraction ").

The rise in titre produced by any cycle will decrease if that cycle overlaps appreciably with the next cycle ; that is, if part of the virus produced in the cycle has started its next phase of multiplication before the whole cycle is completed. This effect should become more pronounced with each successive cycle, until cyclic rise and fall are no longer detectable. Such a smoothing effect might contribute to the difficulty in demonstrating more than one cycle for WS, MEL, and PR8, or more than two for NDV, and probably accounts for the absence of any drop in titre after the first rise of neuro-WS.

With the exception of LEE, all the unadapted strains showed one or two cycles of reproduction and, in the early stages of multiplication, did not appear to differ fundamentally from the adapted strain, neuro-WS. Thus PR8 and neuro-WS had a similar " total-cycle time " (with both strains infectivity started to return after 4 hours), and their behaviour was indistinguishable until the 8th hour or later. However, after the first cycle the unadapted strains showed a failure to continue with the rate of increase promised by the magnitude of their first cycle. This is probably due to a decrease in " average burst size," since, with the exception of MEL, all the unadapted strains show, at the end of their first cycle, titres higher than at any time later (i.e., more virus disappears at the beginning of the second cycle than appears at the end of any subsequent cycle). Such drop in " average burst size " could be due to production of an excess of non-infectious auto-interfering virus, or to production of virus which, although egg-infectious, has intrinsically a low " average burst size " in brain cells. A third possibility is that unadapted strains lack the capacity to spread from the areas in which they are initially deposited ; multiplication would then lead to accumulation in localized areas, with resulting auto-interference and decrease in " average burst size." In contrast to the unadapted strains, neuro-WS shows no evidence of any decline in " average burst size " until a very much higher titre is reached.

The failure to demonstrate multiplication of LEE does not imply that LEE is incapable of any multiplication, or even that considerable multiplication cannot be occurring. Recently Schlesinger (1950), using large inocula, has demonstrated a rise in the titres of haemagglutinin and complement-fixing antigen of PR8 and LEE corresponding to a single cycle only. This cycle was assumed to involve the production of " incomplete " virus only, as no parallel change in infectivity was found. The rise in haemagglutinin was step-wise, there being no appreciable drop from the level reached by the first cycle. From this it

seems likely that, unlike " complete " infectious virus, " incomplete " virus is relatively incapable of becoming undetectable. Schlesinger's failure to detect rise in the infectivity titre of PR8 was probably due to his taking few and widely-spaced samples (at 2, 5–6, 24, and 48 hours).

SUMMARY.

The multiplication, after intracerebral inoculation in mice, of unadapted strains of influenza and Newcastle disease virus was compared with that of an adapted, neurotropic strain, neuro-WS. The difference, as judged by changes in infectivity titre, seemed to lie in the behaviour of unadapted strains during the later stages of their multiplication, rather than in any inherent incapacity of the unadapted strains to undergo cyclic multiplication. Thus, as far as production of infectious virus was concerned, the first reproductive cycle of unadapted PR8 proved indistinguishable both in rapidity and magnitude from that of neuro-WS.

Some of the factors which control the apparent growth of a virus in a tissue are discussed.

I am greatly indebted to all members of this Institute for their continued interest and valuable criticism, and to Miss Mary Cook for preparation of the figures.

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ALKALINE PHOSPHATASE LEVELS IN PLASMA AND LIVER FOLLOWING CARBON TETRACHLORIDE ADMINISTRATION.

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SINCE the discovery (Roberts, 1933) of the increased amount of alkaline phosphatase in the serum in cases of obstructive jaundice, the behaviour of this enzyme in the serum in diseases of the liver has been extensively studied. Histochemical studies on the distribution of alkaline phosphatase activity in the liver damaged by the use of hepatoxic poisons (Wachstein, 1945; Kritzler and Beaubien, 1949; Stowell and Lee, 1950) or experimental biliary obstruction (Wachstein and Zak, 1950) have been carried out to compare such findings in the liver with the level of serum phosphatase. The histochemical techniques have the disadvantage that they give only comparative rather than quantitative data, and absolute values cannot be obtained readily. In the present work an attempt has been made to obtain information about the stability of the liver alkaline phosphatase protein (and the activity) in the face of organ protein catabolism or loss induced by the administration of carbon tetrachloride, and to correlate such findings with the level of alkaline phosphatase in the plasma.

EXPERIMENTAL.

Care and treatment of animals.

Inbred male Wistar strain rats, 11–14 weeks old, weighing 180–250 g. and raised on the laboratory stock diet were used. The stock was composed of the following (g. per 100 g. of the diet): Rice flour 49·6, ground whole wheat 24·0, casein 8·0, fish meal 4·0, whole milk powder 4·0, arachis oil 6·4, shark liver oil 1·6, proteolyzed whole liver powder 1·6, sodium chloride 0·4 and calcium carbonate 0·4). Liver injury was produced by the intraperitoneal administration of carbon tetrachloride (C.P. grade) at a level of 0·5 ml. per kg. every second day, as described by Brauer and Root (1946). Daily food intake of the treated and control animals was recorded. Three groups of animals received 2, 4 and 5 injections. They were sacrificed 48 hours after the last injection. In all experiments food was withdrawn 24 hours before sacrifice. One group of rats which had received 5 injections was allowed to eat *ad libitum* for 120 hours after the last injection and sacrificed after a fasting period of 24 hours. This group served as a reparation experiment. At least one control rat was sacrificed and its liver and plasma analysed along with those from each of the several experimental groups in each day's work. Just before sacrifice all the animals received nembutal intraperitoneally at a level of 80 mg. per kg., and a sample of the blood was withdrawn from the abdominal vein in a heparinized syringe. The liver was rapidly

excised *in toto*, blotted free of blood with filter-paper and chilled in ice-salt mixture.

Methods.

The plasma from the heparinized blood was separated by centrifugation in the cold (2°) at 2000 r.p.m. for 20 minutes. The phosphatase activity was determined on 0·3 ml. aliquots of the plasma in duplicate or triplicate by the method of Bodansky (1937) at 37°, pH 9·2–9·3. The inorganic phosphate liberated was estimated by the method of Fiske and Subbarow (1925) as modified by LePage and Umbreit (1945). In general, the units of phosphatase activity are expressed in terms of mg. of P liberated per 100 ml. of plasma per hour or per g. of liver per hour.

The chilled liver, after removal of the connective tissue, was weighed. The left median lobe was homogenized with cold (0–5°) normal saline for 2 minutes in a test-tube homogenizer of the Potter-Elvehjem (1936) type and diluted to contain 10 per cent by weight of tissue. Aliquots of the homogenate were used for the determination of total nitrogen by the semi-micromethod of Sobel, Yuska and Cohen (1937). The values obtained are assumed to be a measure of the protein content (i.e., per cent of liver protein = per cent of nitrogen × 6·25).

A suitable portion of the homogenate (10 ml.) was spun at 2000 r.p.m. in the cold (2°) for 20 minutes. The fat floating on the top was scooped out, and 0·3 ml. aliquots of the supernatant in triplicate was used for the determination of phosphatase activity (Bodansky, 1937). The supernatant included practically the entire " large granules " of Claude with which much of the liver phosphatase activity is associated.

Another portion of the liver was ground with four times its weight of anhydrous sodium sulphate and extracted with chloroform for 48 hours (Channon, Platt and Smith, 1937). After evaporation of chloroform the extracted material was weighed. This material is hereafter called the " lipid."

RESULTS AND DISCUSSION.

According to Brauer and Root (1946), intraperitoneal administration of carbon tetrachloride at a level of 0·5 ml. per kg. on every second day produces a pronounced liver injury on the fourth day, with little or no change in the degree of the injury on further injections until the tenth day. A prompt and complete recovery ensues from a period of ten days' experimental carbon tetrachloride poisoning following the withdrawal of the drug. Although these authors state that at a level of 0·5 ml. per kg. there is no significant extrahepatic damage, Iyengar (unpublished) in this laboratory has observed marked kidney damage as well. The food intake for the first 4 days of the treatment is on an average 2 g. per day. This would practically amount to a state of inanition. From the 4th day to the 10th day the average food intake rises to 5·3 g. per day, which is still below the normal consumption of 12 g. The food consumption in the restoration experimental group of rats rises rapidly to normal.

From Table I it is apparent that there is a gradual rise in the liver weight per 100 g. body-weight, reaching a maximum after the fifth injection, the increase being about 45 per cent. This observation is in general accord with that of Stowell and Lee (1950), who attribute the increase in weight to greater fluid and

lipid content of the damaged liver. After the fifth injection there is a significant loss of liver protein, whereas the fat content reaches almost double the normal value. The prompt and complete recovery of the biochemical picture and restoration to normality is indicated in Group V, following the withdrawal of the drug.

TABLE I.—*Effect of Carbon Tetrachloride on the Weight, Protein and Lipid of the Livers of Rats.**

Group number.	Treatment.	Number of animals.	Liver weight. (g. per 100 g. body weight.)	Lipid. (g. per cent.)	Protein. (g. per cent.)
I	Control	6	2·19 ± 0·26	7·02 ± 0·99	21·58 ± 0·99
II	2 injections	9	2·40 ± 0·26	10·58 ± 1·72	18·36 ± 1·17
III	4 ,,	11	2·76 ± 0·37	11·37 ± 1·70	17·43 ± 1·72
IV	5 ,,	7	3·18 ± 0·57	13·65 ± 2·08	15·92 ± 1·87
V	144 hours after fifth injection	9	2·08 ± 0·20	7·80 ± 0·89	20·52 ± 1·47

* The data in this and subsequent tables are given with the calculated standard deviation from the mean from the equation, $= \sqrt{(x-x^1)^2/(n-1)}$, where n is equal to the number of rats in a group.

TABLE II.—*Liver Alkaline Phosphatase Activity of Normal and Carbon Tetrachloride-treated Rats.*

Group number.	Treatment.	Number of animals.	Units per g. of liver.	Units per 100 g. initial body weight.	Units per g. of liver protein.	Calculated* units per g. of liver protein.
I	Control	9	1·34 ± 0·12	2·74 ± 0·37	6·72 ± 1·48	..
II	2 injections	9	1·24 ± 0·32	2·61 ± 0·53	6·83 ± 2·02	7·9
III	4 ,,	8	1·27 ± 0·36	2·41 ± 0·59	7·24 ± 1·82	8·3
IV	5 ,,	6	0·80 ± 0·02	2·05 ± 0·16	5·18 ± 1·22	9·1
V	144 hours after fifth injection	10	1·20 ± 0·21	2·43 ± 0·52	5·85 ± 1·24	7·1

* Calculation of expected mean activity on the assumption that none of the initial enzyme activity is lost and that enzyme protein is an insignificantly small part of the total protein.

Table II presents a comparison of the liver alkaline phosphatase activity between the various groups of animals. The phosphatase activity is expressed in terms of units per 100 g. body-weight and in terms of units per g. of liver protein. These methods of comparison eliminate variations in figures per unit g. of liver as a result of dilution due to glycogen or lipid. Representation of results in units per 100 g. of body-weight is desirable as being a method of comparing total activity in animals of different weights, and is also representative of the total enzyme activity for a given animal. In the carbon tetrachloride-treated animals the initial body-weight is used for this calculation because it is comparable to the initial weight of liver from which losses have occurred. The enzyme activity in units per g. of liver protein allows a direct comparison of the loss of enzyme activity with the loss of liver protein in general. From Table II it is clear that there is a gradual decline in the phosphatase activity per g. of tissue in Groups II, III and IV compared to normal, and a return to almost original level in Group V. This may be attributed to a dilution effect as the

liver weight increases following the damage by carbon tetrachloride. The changes in the total activity when expressed in units per 100 g. body-weight are not markedly different between the treated and control groups of animals. It appears that on a basis of units per g. of liver protein there is some preferential conservation of the phosphatase activity in the face of the organ protein loss. However, such preferential conservation seems incomplete, when the activity found in units per g. of liver protein is compared with the activity calculated on the assumption that the enzyme protein (and the activity) is spared while protein in general is lost. The interpretation of the changes in the liver phosphatase activity in these experiments involves the assumption that the enzyme activity as measured is proportional to the enzyme protein *per se*. The validity of this assumption has been carefully examined and largely justified by Miller (1948) in a comparable investigation. It may also be pointed out that the interpretation of the changes in the alkaline phosphatase in the liver in this investigation is complicated by the fact that the contribution of the biliary tract epithelium to the alkaline phosphatase activity of a liver homogenate is real, but remains an unevaluated factor.

TABLE III.—*Plasma Alkaline Phosphatase Activity of Normal and Carbon Tetrachloride-treated Rats.*

Group number.	Treatment.	Number of animals.	Units per 100 ml.
I	Controls	4	12·2 ± 1·92
II	2 injections	3	8·8 ± 1·19
III	4 ,,	7	17·82 ± 2·92
IV	5 ,,	3	27·81 ± 1·92
V	144 hours after the fifth injection	8	11·41 ± 1·14

Table III shows the changes in the plasma phosphatase activity of the control and treated animals. After two injections there is a significant drop in the plasma phosphatase activity. Either this might be due to an actual decrease in the amount of enzyme present in the blood plasma, or substances which are inhibitors of phosphatase activity might appear in the plasma as a consequence of carbon tetrachloride treatment. A direct effect of either carbon tetrachloride or the products of its metabolism upon the plasma phosphatase activity can be eliminated for two reasons. Firstly, such inhibitory effects are absent after the fourth and fifth injections. Secondly, in preliminary experiments it was found that the resulting phosphatase activity of suitable admixtures of plasma from Group I and II was equal, within experimental error, to the activity calculated from the contribution of each component. Several investigators have examined the alkaline phosphatase content of blood serum under different dietary conditions. In starvation the amount of serum or plasma alkaline phosphatase decreases (Bodansky and Jaffe, 1931 ; Oppenheimer and Flock, 1947). The fall in the plasma phosphatase activity observed after two injections appears to be largely due to the effect of starvation, as the animals are practically in a state of inanition during this period. After the fourth and the fifth injections the plasma phosphatase activity rises sharply, in conformity with the observation of other workers

(Hartmann and Schelling, 1934; Freeman, Chen and Ivy, 1938) on hepatic damage induced by carbon tetrachloride. In the restoration experiment (Group V) the plasma phosphatase activity returns practically to normal values, indicating the reversible nature of the liver damage.

The role of liver in phosphatase metabolism has been demonstrated by three different laboratories with identical results. After hepatectomy Armstrong and Banting (1935), Maddock, Schmidt and Thanhauser (1942) and Dalgaard (1949) found a rise in serum phosphatase, demonstrating the participation of liver in the regulation of the enzyme level in blood. Evidence is available to show that apart from the liver, the intestinal tract (Kosman, Kalbersz and Freeman, 1943) and the pancreas (Freeman and Ivy, 1937; Nothmann, 1944) may also play a role in the removal of phosphatase from the blood. Two theories (Moog, 1946) have been put forward to explain the role of liver in phosphatase metabolism. According to one, phosphatase is produced by various organs, including the liver. In liver damage or biliary obstruction the phosphatase formed by the liver cells, instead of being excreted in the bile, is turned back into the blood stream. This explanation apparently implies an increased phosphatase synthesis by the liver cells. The results of the present investigation show that, although there appears to be some preferential conservation of the enzyme protein in the face of organ protein loss, there is no evidence of increased synthesis of phosphatase by the liver cells. The second theory postulates that the rise of plasma phosphatase following liver damage or biliary obstruction is due to the inability of the liver to excrete the enzyme that is formed by various organs, particularly by bone (Gutman and Jones, 1949). On the basis of evidence presented in this paper, it appears that in liver damage the ability of the organ to excrete the surplus enzyme is affected, thus favouring the second hypothesis.

SUMMARY.

1. Administration of carbon tetrachloride intraperitoneally at a level of 0·5 ml. per kg. every second day results in a significant rise in liver weight per 100 g. body-weight, loss of liver protein and increase in liver lipid after the fifth injection. On stopping the injections the biochemical picture quickly returns almost to normal.

2. In the face of liver protein loss there appears to be some conservation of the liver alkaline phosphatase protein (and the activity), although this conservation is not complete. After the second injection there is a fall in the plasma phosphatase activity, probably due to a starvation effect. Following the fourth and fifth injections there is a sharp rise in the plasma phosphatase activity, which however returns to normal after the subsequent withdrawal of the drug.

3. The results support the idea that the role of liver in phosphatase metabolism is primarily that of a regulatory organ concerned with the excretion of surplus phosphatase.

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A POSSIBLE DELETION IN A HUMAN RH CHROMOSOME: A SEROLOGICAL AND GENETICAL STUDY.

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A SAMPLE of serum from Mrs. B—, tested at the Bristol Blood Transfusion Service, was found to agglutinate all of 1400 red cell suspensions, but it did not agglutinate Mrs. B—'s own red cells. We were very kindly given samples of this blood to investigate further, and a brief report has already been made (Race, Sanger and Selwyn, 1950). Both the serum and the red cells are possibly unique; the combination of the two must surely be so.

The investigation of Mrs. B—'s blood took place during her third pregnancy; her first child was stillborn, death being thought to be connected with a very prolonged labour associated with placenta praevia. The second child died when 12 hours old of atelectasis and prematurity associated with mild haemolytic disease (the direct anti-globulin test was positive). The third child died of haemolytic disease a few minutes after being born. Mrs. B— has never been given a transfusion nor, as far as is known, any injection of blood.

THE RED CELLS OF MRS. B—.

The blood groups of Mrs. B— are O, NsNs, P+, —D—/—D—, Lu(a—), K—, Le(a—) and Fy(a+). Outside the Rh system we could find nothing abnormal in any of these groups. The direct anti-globulin test on the cells was negative.

The Rh genotype at first presented a formidable puzzle; the cells were agglutinated in saline by all the anti-D sera with which they were tested, but by only some of the anti-E and by most but not all of the anti-C. As the cells did not react with anti-c nor with anti-e it at first seemed that they must have peculiar forms of both the E and the C antigens, and moreover that they must be homozygous for these forms. As will be seen in the sections dealing with the individual antigens, this problem was ultimately resolved when it was realized that the cells were unique in that they were agglutinable *in saline* by incomplete anti-D. It was the incomplete anti-D in certain anti-E and anti-C sera that was giving the false impression of the presence of peculiar forms of E and C antigens. In fact, as the following sections show, the Ee and Cc allelomorphic series of antigens were not represented, while the D antigen was unusually powerful. The curious fact that the cells were homozygous for such a rare peculiarity was explained when inquiry established that the parents of Mrs. B— were half second cousins.

The mother of Mrs. B— is recognizably heterozygous for the unusual chromosome, her genotype being —D—/CDe. In the Tables the reactions of these cells are shown together with those of Mrs. B—'s cells whenever comparable samples were tested.

The antigen D.

Mrs. B—'s cells were strongly agglutinated in saline by the 6 anti-D sera with which they were tested. The results of titrations in saline of these anti-D sera are shown in Table I. In this and the following experiments where quantitative comparisons have been made the cell suspensions were matched by eye in uniform tubes of narrow bore; they were made from clots taken by venepuncture from all the donors at the same time.

The total scores in Table I do not show clearly the greater amount of D in —D—/—D— blood, which is demonstrated by other methods. For example, Table II shows how one of these anti-D sera (Mol.) makes a sharp distinction in albumin which it does not make in saline. The greater amount of D is well

TABLE I.—*Showing that —D—/—D— Cells are Strongly Agglutinated by Anti-D Sera.*

Red cells.		Anti-D sera, titrations in saline. Wo.	Hu.	Wes.	Mol.	P.A.	P.B.	Total scores.
Propositus	—D—/—D—	67	60	73	78	75	69	422
Mother of propositus	—D—/CDe	65	62	73	76	76	68	420
Control	CDe/cDE	59	56	65	75	65	68	388
,,	CDe/cde	60	56	57	73	62	63	371

The figures show the scores achieved by the various cell samples when tested against titrations of antisera. The six symbols by which we distinguish different strengths of agglutination score as follows: +++ = 10, ++ = 8, + = 5, (+) = 3, w = 2, and − = 0. For example a titration result 1/1 +++, 1/2 +++, 1/4 ++, 1/8 +, 1/16 (+), 1/32 w, 1/64 − would score 38.

TABLE II.—*Showing that —D—/—D— Cells Absorb more Anti-D from the Serum Mol. than do CDe/cDE Cells.*

Red cells		Anti-D serum Mol. Unabsorbed.	Absorbed by —D—/—D—.	Absorbed by CDe/cDE.
		Titrations in saline.		
Propositus	—D—/—D—	77	35	45
Control	CDe/cDE	74	30	50
		Titrations in albumin.		
Propositus	—D—/—D—	79	18	33
Control	CDe/cDE	55	15	25

For scoring system see Table I.

shown by absorption tests (Table II); —D—/—D— cells remove more anti-D than do the control CDe/cDE cells.

Table III shows that —D—/—D— cells are more efficient than CDe/cDE cells in absorbing incomplete anti-D.

The exaltation of D in the red cells of the propositus and, to a lesser degree, in the red cells of her mother, is shown most clearly by their unique ability to be

TABLE III.—*Showing that —D—/—D— Cells are More Efficient in Absorbing Incomplete Anti-D than are CDe/cDE Cells.*

Red cells.		Incomplete anti-D serum Mah. Unabsorbed.	Absorbed by —D—/—D—.	Absorbed by CDe/cDE.
		Titrations in albumin.		
Propositus	—D—/—D—	123	73	95
Control	CDe/cDE	123	68	96

For scoring system see Table I.

TABLE IV.—*Showing that —D—/—D— Cells are agglutinated in Saline by Incomplete Anti-D.*

Red cells.		Incomplete anti-D sera. Titrations in saline. Wil.	Ire.	Dou.	Mah.	For.	Rey.*
Propositus	—D—/—D—	9	25	30	36	20	23
Mother of propositus	—D—/CDe	2	4	0	2	0	10
Control	CDe/cDE	0	0	0	0	0	..
,,	CDe/cde	0	0	0	0	0	0

* Also contains anti-E.
For scoring system see Table I.

agglutinated by incomplete anti-D *in saline* (Table IV). Altogether, the cells of the propositus were agglutinated in saline by 14 out of 16 incomplete anti-D sera (not counting those containing anti-C or anti-E as well).

In brief, the tests described all agree in showing that the —D—/—D— cells contain more D antigen than do normal homozygous DD cells. The curious ability of these cells to be agglutinated in saline by incomplete anti-D suggests that the incomplete antibody is not monovalent as it is so frequently represented. It suggests rather that some hindrance to agglutination in saline by incomplete anti-D is removed when all C and E antigens are absent from the cells.

The antigen C.

It has already been mentioned that the red cells of Mrs. B— were agglutinated by most anti-C sera. Later it was realized that the reaction was due to the ability of her cells to be agglutinated by incomplete anti-D in saline. The anti-C sera which failed to agglutinate her cells were those containing anti-C, but no incomplete anti-D (Table V). The four negative reactions were confirmed by indirect anti-globulin tests, by trypsin tests and by tests in albumin.

Another way of demonstrating that it is the presence of incomplete anti-D in anti-C sera that causes the agglutination of —D—/—D— cells may be seen from the first and last columns of Table V. The serum Ba. (1st column) contains a good deal of incomplete anti-D, but when this is removed by repeated absorptions with cDe/cde cells (last column), and only anti-C remains in the serum, it no longer agglutinates the —D—/—D— cells.

TABLE V.—*Showing that —D—/—D— Cells are Not Agglutinated by Anti-C Sera Free from Incomplete Anti-D.*

Red cells.		Anti-C sera. With incomplete anti-D. Titrations in saline.								Without incomplete anti-D. Titrations in saline or albumin.			
		Ba.	Hi.	Pi.	Ho.	Br.	Fa.	Wa.	Re.	And.	Mar.	Ed.	Ba. abs.
Propositus	—D—/—D—	43	57	24	41	41	+	+	+	0	0	0	0
Mother of propositus	—D—/CDe	55	60	13	54	15	+	+	+	21	..	..	..
Control	CDe/cde	53	58	12	62	6	+	+	+	20	37	37	38

For scoring system see Table I.

TABLE VI.—*Showing that —D—/—D— Cells do not Absorb Anti-C.*

Red cells.	Anti-C serum And. (No incomplete anti-D.) Titrations in saline.	
	Unabsorbed.	Absorbed by —D—/—D—.
CDe/cde	17	22

Red cells.	Incomplete anti-C serum Mar. (no incomplete anti-D). Titrations in albumin.			
	Unabsorbed.	Absorbed by —D—/—D—.	Absorbed by cDE/cDE.	Absorbed by CDe/cDE.
CDe/cDE	37	37	38	3

For scoring system see Table I.

TABLE VII.—*Showing that —D—/—D— Cells are Not Agglutinated by Anti-c Sera.*

Red cells.		Anti-c sera. Anti-c. Titrations in saline.		Incomplete anti-c. Titrations in albumin.			
		Wes.	Ste.	St. L.	Wig.	Ket.	Joh.
Propositus	—D—/—D—	0	5*	0	0	0	0
Mother of propositus	—D—/CDe	0	5*	0	0	0	0
Control	CDe/cde	23	24	25	23	35	40
,,	cde/cde	60	67	43	53	..	..
,,	CDe/CDe	0	2*	0	0	0	0

* For explanation see text.
For scoring system see Table I.

TABLE VIII.—*Showing that —D—/—D— Cells do not Absorb Anti-c.*

Anti-c sera.	Titration scores against CDe/cDE cells. Unabsorbed.	Absorbed by —D—/—D—.	Absorbed by CDe/cDE.	Absorbed by CDe/CDe.
Wes.	70	70	49	70
Wig.	33	40	10	37
St.L.	31	38	18	33
Ket.	48	40	18	38

For scoring system see Table I.

TABLE IX.—*Showing that —D—/—D— Cells do Not Absorb Anti-E.*

Red cells.	Anti-E serum Rey. (containing incomplete anti-D). Unabsorbed.	Absorbed by —D—/—D—.	Absorbed by CDe/cDE.	Absorbed by CDe/cde.	Absorbed by cde/cde.
	Titrations in saline.				
Propositus —D—/—D—	22	0	0	0	18
Mother of propositus —D—/CDe	10	0	0	0	5
Control CDe/cDE	53	50	5	54	57
,, CDe/CDe	0	0	0	0	0

Red cells.	Anti-E serum Mad. (no incomplete anti-D). Unabsorbed.	Absorbed by —D—/—D—.	Absorbed by CDe/cDE.	Absorbed by CDe/cde.
	Titrations in saline.			
CDe/cDE	25	27	0	23

For scoring system see Table I.

TABLE X.—*Showing that —D—/—D— Cells do Not Absorb Anti-e.*

Red cells.	Anti-e serum Bru. Unabsorbed.	Absorbed by —D—/—D—.	Absorbed by CDe/cde.	Absorbed by cDE/cDE.
	Titrations in saline.			
CDe/cDE	55	57	9	51

For scoring system see Table I.

TABLE XI.—*The Blood Groups of Mrs. B— and of Other Members of Her Family.*

	Rh.	ABO.	MNS.	P.	Lu(a).	K.	Le(a).	Fy(a).
Mrs. B—	—D—/—D—	O	NsNs	+	—	—	—	+
Mr. B—	CDe/cde	B	MsNs	+	—	—	—	+
Baby B—	—D—/CDe	B	MsNs	+	—	—	—	+
Mrs. B—'s mother	—D—/CDe	O	MsNs	+	—	—	+	+
Mrs. B—'s half brother	CDe/cde	B	MSMs	+	—	—	—	+
Mrs. B—'s cousin	cDE/cde	O	MN.S	+	—	—	—	+
Mrs. B—'s cousin's 1st child	cDE/cde	O	MM.S	+	—	—	+	+
,, ,, ,, 2nd child	cDE/cde	A_1	MM.S	+	—	—	—	+

It is also worth noting, though it is not shown in the table, that the strength of reaction of the first five sera in Table V with —D—/—D— cells is in proportion to the amount of incomplete anti-D in these sera (as judged by titrations in albumin against cDe/cde cells), and not in proportion to the amount of anti-C (as judged by titrations in saline against Cde/cde cells).

Finally, the —D—/—D— cells do not absorb anti-C (Table VI).

The antigen C^w.

The red cells of Mrs. B— were not agglutinated by any of four anti-C^w sera (Lo., Du., M., and Mo., of which the first two are extremely powerful examples). Absorption tests were not done.

The antigen c.

Mrs. B—'s cells do not possess the antigen c. Table VII shows the results of titrations in saline with two anti-c sera, and of titrations in albumin with four incomplete anti-c sera. All the negative reactions with the —D—/—D— cells and the —D—/CDe cells were confirmed by indirect anti-globulin tests. It will be seen that one anti-c serum (Ste.) weakly agglutinated —D—/—D—, —D—/CDe and the control CDe/CDe cells in saline. The indirect anti-globulin test was negative with these three cell samples, and after a small absorption with CDe/CDe cells the serum no longer agglutinated —D—/—D— or —D—/CDe cells. It was assumed that the serum contained a small amount of some other agglutinin for these cells.

The absence of the antigen c was confirmed by the complete failure of —D—/—D— cells to absorb anti-c (Table VIII).

The antigen E.

The red cells of Mrs. B— were tested with ten anti-E sera. Seven of these anti-E sera were from Rh-positive donors and therefore did not contain incomplete anti-D. Mrs. B—'s cells were negative with all seven when tested in saline, in albumin, with trypsin and by the indirect anti-globulin method. Three of the anti-E sera were from Rh-negative donors and contained incomplete anti-D; Mrs. B—'s cells were agglutinated by the incomplete anti-D in these sera. Table IX shows that absorption of one of these three sera (Rey.) by CDe/cde cells prevents this positive reaction while absorption with cde/cde cells does not.

The absence of the antigen E was confirmed by the failure of —D—/—D— cells to absorb anti-E (Table IX).

The antigen e.

The antigen e is not present in Mrs. B—'s cells. Only one anti-e serum, Bru., was available, not counting the anti-e present in Mrs. B—'s own serum. (Mrs. B—'s serum did not agglutinate her own cells by any of the known tests.) Mrs. B—'s cells did not react with the anti-e serum Bru. when tested in saline, in albumin, with trypsin or by the indirect anti-globulin method.

The absence of the antigen e was also clearly demonstrated by the failure of —D—/—D— cells to absorb anti-e (Table X).

The antigens c^v, C^u and E^u.

No antisera are known specific for these antigens, which are recognized by their reacting with some, but not all anti-C and anti-E sera respectively.

Most of the anti-C sera used in the present investigation had been used in the work on the allelomorphs c^v and C^u (Race, Sanger and Lawler, 1948; Race and Sanger, 1951). The presence of these antigens in Mrs. B—'s cells could therefore be ruled out.

Three of the anti-E sera used were those known to be capable of agglutinating E^u cells (Ceppellini, Ikin and Mourant, 1950) so the presence of this antigen was also excluded.

THE SERUM OF MRS. B—.

The serum of Mrs. B—, when investigated at the Bristol Blood Transfusion Service, had been found to agglutinate all of 1400 blood samples.

A few preliminary titrations showed that the reactions in saline were strongest at 37° C. and decreased through 25° C. and 18° C. to 4° C., at which temperature the titre was about 1 in 16. At 37° C. the titre was about 1 in 128 whether the titrations and the cell suspensions were in saline or in 20 per cent albumin. Mrs. B—'s cells were not agglutinated by her own serum, nor did her cells give a positive direct, or indirect, anti-globulin test.

Since the case-history was so typical of haemolytic disease it seemed likely that the responsible antibody or antibodies would belong to the Rh or Kell systems.

In the hope of separating anti-Rh antibodies that might be present, separate samples of the serum were absorbed by cDE/cDE cells, by CDe/CDe cells, by cde/cde cells and by CDe/cDE cells.

Anti-e.

The serum of Mrs. B— was absorbed with an equal quantity of packed O, cDE/cDE red cells. Some of it was kept and the remainder absorbed a second time, and so on to four times. The three times and four times absorbed samples no longer agglutinated the absorbing cells; they were shown to contain anti-e when they agglutinated all of 18 e-containing blood samples, but failed to agglutinate all of 6 cDE/cDE samples. The probability of obtaining such a correspondence merely by chance is 1 in 135,000.

At this stage it was noticed that the absorbed serum was giving the expected dosage effect with cde/cde and cDE/cd cells; the ee cells scoring more highly than the Ee. But it was also noticed that when C was present the dosage effect of anti-e was upset, for CDe/cde and CDe/CDe scored more highly than cde/cde, and CDe/DE more highly cDE/cde. This strongly suggested that anti-C was present in the serum as well as anti-e.

Anti-C.

The samples of Mrs. B—'s serum which had been absorbed with cde/cde cells were then examined. One of them, absorbed three times with an equal volume of packed cells and a fourth time with a half volume, was satisfactory for a clear

demonstration of anti-C ; it was tested against 24 cell samples with these results :

		Red cells. C.	Red cells. No C.
Serum of Mrs. B— absorbed by cde/cde $3\frac{1}{2}$ times .	+	14	1
	—	1	8

The probability of getting this distribution by chance is 1 in 10,000. Furthermore the two wrong results became right on repeating the tests. The anti-C was very weak after the amount of absorption necessary to remove the other antibodies. That this was the result of cross-absorption is probable, for the anti-C which was interfering with the dosage effect of anti-e was powerful.

Anti-c.

On investigating the samples of Mrs. B—'s serum which had been absorbed by CDe/CDe cells the following results of agglutination tests in saline made it all but certain that anti-c was present :

		Red cells. c.	Red cells. No c.
Serum of Mrs. B— absorbed by CDe/CDe 3 times .	+	8	0
	—	1	3

The probability of getting this distribution by chance is 1 in 55. However, the existence of anti-c was proved when a sample of the serum absorbed $2\frac{1}{2}$ times by CDe/CDe cells gave the following indirect anti-globulin test results :

		Red cells. c.	Red cells. No c.
Serum of Mrs. B— absorbed by CDe/CDe $2\frac{1}{2}$ times .	+	9	0
	—	0	6

for the probability of getting this distribution by chance is but 1 in 5000.

The anti-c in the absorbed serum was weak. This, again, must have been the result of cross-absorption, for the anti-c in the unabsorbed serum was powerful, as judged by its reaction with cDE/cDE cells (which reaction could not be attributed to the anti-e nor to the anti-C present in the unabsorbed serum).

Comments on the Antibodies.

The sample of serum in which the antibodies anti-C, anti-c and anti-e were present was taken from Mrs B— after her second delivery but before her third conception. The groups of the first two children are, unfortunately, not known ; they could have been —D—/CDe and —D—/cde, and the antibodies may have been straightforward specific responses to the foetal antigens C, c and e.

This explanation is not put forward with confidence, for it is unusual for a strong antibody to be made during a first pregnancy, as either the anti-C or the anti-c must have been if we are to believe that the immune response was simple and specific. Furthermore, the great difficulty of splitting the anti-C from the anti-c, contrasted with the relative ease of splitting the anti-e from the other two antibodies, also leads us to consider another possible manner of origin for the anti-C and anti-c.

Race, Sanger and Lawler (1948) dallied with the idea of a widening of scope of antibody response when faced with the facts that the antibody made against C can stray as far beyond anti-C as anti-C^w and anti-c^v, and that the antibody made against c can stray as far beyond anti-c as anti-c^v.

Is it possible that in ordinary people the presence of the allelomorph of the immunizing antigen exerts a restraining canalizing effect on the antibody response ? In a person like Mrs. B—, with no C nor c antigen, there may be nothing to prevent the antibody response to one or the other antigen, C or c, spreading from anti-C to anti-c. If, however, such a thing is possible, then the response of Mrs. B— to the antigen e might be expected to be anti-e and anti-E. We have not been able to demonstrate the presence of anti-E in the serum though, if it had originated in the way suggested, its demonstration would probably be difficult.

It has not been possible to demonstrate the presence of anti-d in the serum. Samples of serum taken during the third pregnancy appeared to have antibodies of the same specificity and of about the same strength as those in the sample most exhaustively examined, which was taken before the third pregnancy began.

Impossibility of Finding a Compatible Donor for Mrs. B—.

It is perhaps of interest to note that the antibodies anti-e, anti-C and anti-c were recognized in the serum of Mrs. B— during her third pregnancy, and the problem arose of finding compatible blood for her should she need it (her first pregnancy had been complicated by placenta praevia). There was clearly no hope of finding a donor in the general population ; it is quite possible that no blood in the world would be compatible with these three antibodies. The two brothers of Mrs. B— each had a 1 in 4 chance of being —D—/—D—, but both were dead. The only hope, therefore, of finding a donor for Mrs. B— seemed to lie in the offspring of consanguineous marriages in her family. The pedigree (Fig. 1) shows that two boys were in this category, though the hope of their being —D—/—D— was rather forlorn, each boy having but 1 chance in 256. In fact neither boy had received even one —D— chromosome.

It was inevitable that Mrs. B—'s third child would suffer from haemolytic disease and would probably require transfusion. With this in view, and the search for a donor having failed, it was planned to take blood from Mrs. B— herself a few days before delivery, and to wash the cells free from antibody before transfusing the baby, but Mrs. B— spontaneously started labour four weeks prematurely. The baby was delivered uneventfully, showing signs of severe haemolytic disease with a cord blood haemoglobin concentration of 3·5 g. per 100 ml., and died in a few minutes. The mother fortunately did not need a transfusion.

OTHER MEMBERS OF MRS. B—'S FAMILY.

The Rh groups of Mrs. B— and other members of the family are shown in the pedigree (Fig. 1). Mrs. B—, the propositus, is marked with an arrow. The pedigree shows how Mrs. B—'s parents are half second cousins, and how she comes to be homozygous for the —D— chromosome.

The full blood groups of the family are shown in Table XI. Details of the reactions of the red cells of Mrs. B—'s mother, who is —D—/CDe, have already

been given in Tables I, IV, V, VII and IX. It will be seen from Table IV that the heterozygous —D— cells are agglutinated by incomplete anti-D in saline, although nothing like so strongly as homozygous —D— cells. With the exception of Mrs. B—'s baby no further members of the family so far tested have other than normal groups (Table XI).

A sample of the cord blood of the dead baby was available. The cells gave a strongly positive direct anti-globulin test, due to the presence of maternal antibody. This at first obscured the Rh groups of the baby. After washing the baby's cells four times in large volumes of warm saline (between 56 and 60° C.)

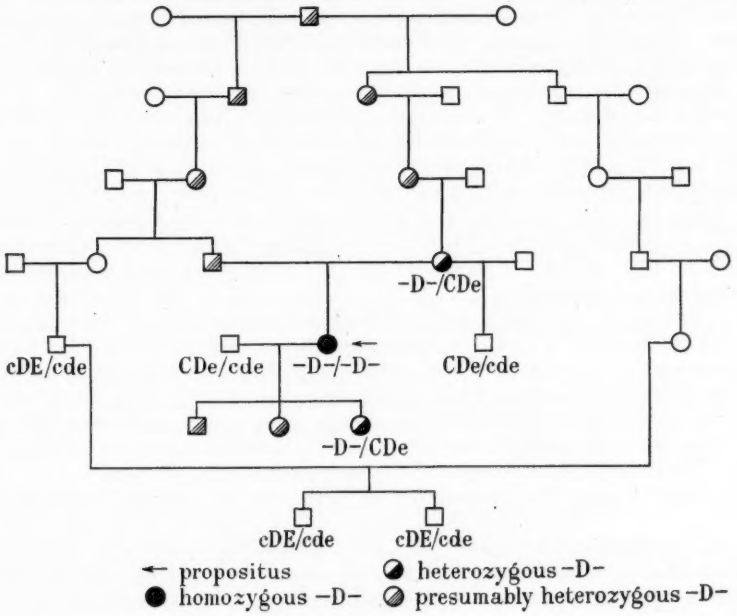

FIG. 1.

the direct anti-globulin test became negative and the Rh groups could be determined without difficulty (and, incidentally, so could the Kell and Duffy groups, which depend on an indirect anti-globulin test). The Rh genotype was —D—/CDe, and again the cells were agglutinated in saline by incomplete anti-D though more weakly than homozygous —D— cells. The antibody which could be eluted from the baby's cells and that present in the baby's serum gave reactions parallel with the antibody in Mrs. B—'s serum, although much weaker. No attempt was made to separate the antibodies in the baby's serum.

Mrs. B—'s father is dead; he and other presumptive —D— heterozygotes are indicated in the pedigree (Fig. 1) by hatching. The pedigree also shows that the unusual chromosome must almost certainly have originated at least five generations back in Mrs. B—'s ancestry, that is, before 1800.

THE GENETICAL INTERPRETATION.

It seems that there are several possible genetical interpretations of the antigenic content of Mrs. B—'s red cells.

1. The C and E loci may be represented, but by unknown silent allelomorphs. This is perhaps unlikely, because all the known allelomorphs produce antigens that are easily detectable ; it is highly improbable because two of this new kind of silent allelomorph, one at the C and the other at the E locus, would have to be postulated on the same chromosome.

2. A modifying gene may be at work. It is difficult to imagine that such a gene would suppress all recognizable effects of the C and E genes, yet not of the D genes. Moreover, even the presence of modified C or c antigens would surely be incompatible with the existence in the serum of both anti-C and anti-c.

3. Deletion of a portion of the Rh chromosome seems to us to offer the most satisfactory explanation. A deletion would fit well with the total absence of the C and E antigens and could explain the exalted D antigen. There are facts (Lawler and Race, 1950) which can be interpreted as evidence for the existence of a common basic material which is needed in the development of the CDE antigens. There appears to be competition for this substance, which suggests that it is available in limited amount rather than in excess. Freed from the competition of any kind of C and E, D might be able, for once, to quench its need of this hypothetical raw material.

If a deletion is responsible then it is probably a short one, for Mrs. B— enjoys good health, and has no other apparent congenital abnormality.

Whatever the exact genetic mechanism may be, the fact is that C and E have together been overwhelmed, while D remains triumphant. This supports, from an unexpected angle, Fisher's tentative suggestion of five years ago (Fisher and Race, 1946) that the order of the genes on the chromosome would be found to be DCE. The order DCE requires only one accident to produce Mrs. B—'s chromosome, while the order CDE would appear to require two separate catastrophes to do so.

It was pointed out by Fisher and Race (1946) that there was some indirect evidence that the CDE genes, though obviously tightly linked, were occasionally separable. The separation of the C and E antigens from the D antigen in the blood of Mrs. B— must surely reflect a separability of the genes.

SUMMARY.

A sample of blood is described which is possibly unique both in the Rh antigens of its red cells and in the Rh antibodies of its serum.

The red cells lack any detectable representative of the C and of the E allelomorphic series of antigens, although D is present and in more than usual amount. The genotype can be represented thus : —D—/—D—. These red cells have the property, not before encountered, of being agglutinable *in saline*, by incomplete anti-D. This suggests that incomplete anti-D is not monovalent, as it is so often represented. That the donor is homozygous for such an extraordinary chromosome is undoubtedly due to her parents having been half second cousins.

The serum contains the Rh antibodies anti-e, anti-C and anti-c. These antibodies are presumably the result of immunization by pregnancy, for the donor

has never been transfused and her second and third children died of haemolytic disease.

Seven other members of the family have been tested; two of them, the mother and the child of the propositus, were heterozygous for the unusual chromosome (—D—/CDe). It is possible to recognize the chromosome —D— in single dose by the ability of the red cells to be agglutinated in saline by incomplete anti-D, although the reaction is weaker than that with the homozygous cells.

The most likely genetic explanation of the curious antigenic structure is that the C and E genes have been involved in a small deletion. The deletion could hardly be a large one because of the good health of the donor, and because of her freedom from any other apparent congenital abnormality.

The fact that C and E and not D are involved supports the tentative suggestion made by Fisher, five years ago, that the order of the genes would be found to be DCE. Further, this separation of the C and E antigens from the antigen D must surely reflect a separability of the genes.

We wish to thank the members of this family for their co-operation. We are particularly grateful to Mrs. B—, who gave numerous samples of blood most willingly and furnished the pedigree shown in Fig. 1. Our thanks are also due to Dr. J. Campbell Gordon, the obstetrician in charge of Mrs. B—, for his kind co-operation.

Once again we wish to acknowledge our indebtedness to Dr. A. E. Mourant, of the Medical Research Council Blood Group Reference Laboratory, for most of the antisera used in the investigation.

Finally, for the pleasure of working on this problem, we are extremely grateful to Dr. G. H. Tovey and his staff at the South-West Regional Transfusion Centre, and to Dr. Mourant and Miss Elizabeth W. Ikin.

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THE BEHAVIOUR *IN VIVO* OF PARTICULATE MICROCOCCIN.

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THE possibility of using a particulate antibiotic for the treatment of tuberculosis has been discussed by Markham and Florey (1951). In this paper the behaviour in the animal body of an almost insoluble antibiotic, micrococcin (Su, 1948), is described. The experiments were undertaken as a preliminary to an investigation of its effect on experimental tuberculosis (Markham, Wells, Heatley and Florey, 1951).

EXPERIMENTAL.

Preparation of a Suspension of Micrococcin.

Before any animal experiments could be conducted it was necessary to have a fine, stable, sterile suspension of micrococcin which would not agglutinate when introduced into the blood stream, and would not contain particles large enough to cause blockage of blood vessels. After unsuccessful attempts with mechanical trituration and ultrasonic vibration, a uniformly fine suspension was obtained when water was added forcibly to an alcoholic solution. The alcohol could not safely be removed by distillation, and the suspended particles were too fine to be centrifuged down. However, the addition of a small amount of sodium chloride caused sufficient flocculation to permit centrifuging of the particles without preventing their subsequent re-dispersion in water. Although such suspensions could be mixed with normal saline and sometimes injected without harm, in all but the earliest experiments they were stabilized by the presence in the final suspension of about one-third of the volume of serum of the animal species being studied.

The routine procedure for preparing sterile suspensions was as follows :

A solution of about 1 g. of crystallized micrococcin in 25 ml. of 90 per cent alcohol was placed in a sterile 250 ml. centrifuge bottle. To it were added forcibly about 150 ml. of sterile water and 10–15 ml. of sterile normal saline. The suspension was centrifuged and the supernatant was discarded. The sediment was re-suspended in about 100 ml. of water by sucking it backwards and forwards in the " sampling device " (Heatley, 1950), which was also used for adding sterile water and saline, and for removing supernatants. After centrifuging and discarding the supernatant once more, the sediment was suspended in a known volume of serum (say 16 ml.) by sucking back and forth in the pipette, and the total volume of the suspension was measured ; this is the most practicable way of arriving at the volume of the packed sediment, which was usually about 15 ml. but which varied slightly from time to time. The appropriate volumes of saline and water were then added and the suspension was distributed into small sterile screw-capped phials, which were shaken for a few minutes in a rapid shaker or in the Mickle (1948) vibrator, then centrifuged at the slowest possible speed (30–35 $\times$ *g*) for 4 minutes to remove any large particles. The

* Nuffield Dominions Demonstrator in Pathology.

supernatant was cautiously removed with a Pasteur pipette and placed in another sterile capped phial, while the amount of micrococcin left behind was estimated fluorimetrically, and the concentration of the suspension re-calculated accordingly. These suspensions could be kept for weeks in the refrigerator; they were briefly shaken just before use. All fluids were glass-filtered before sterilization to remove dust, etc., and the caps of the phials were carefully cleaned to remove loose lacquer, an unsuspected source of emboli.

The particles of such a suspension appeared to be uniformly small and all exhibited vigorous Brownian movement.

Toxicity.

Preliminary experiments with suspensions of highly purified but not crystalline material containing no stabilizer, such as serum, showed that no reaction occurred in mice following the intravenous injection of relatively small amounts (e.g., 1 mg.). These experiments will not be described here, as it was found that a fine suspension of purer material such as that just mentioned could be injected in much larger amounts than were at first thought possible.

All the mice used for toxicity tests weighed between 18 and 20 g. at the start of the experiments.

Single doses.—Two mice were each given 24·6 mg. intravenously in 1·0 ml. of serum-saline mixture, the time of injection being about 4 minutes. They both looked sick for about 12 hours, and one only revived after receiving subcutaneous injections of saline. Both eventually recovered and put on weight normally. When killed 71 and 335 days later both were in good health.

At a later date 5 mice were injected with 25 mg. in 1 ml. of serum-saline, but of these only one survived, the others dying in about 3 hours. Five mice that were each given 12·5 mg. of the same preparation were slightly subdued after the injection but recovered their activity fully. Possibly the symptoms and death following the injection of about 25 mg. in a serum-saline suspension were due to the blocking of some of the main blood vessels by emboli rather than to a direct toxic effect.

On many occasions doses of 5 mg. in 0·5 ml. were given intravenously without any observable effect on the mice. If however no stabilizer such as serum was present in the suspension the clumps of micrococcin present acted as emboli, and doses as small as 5 mg. then sometimes caused death.

Repeated injections.—In the first series 4 female mice weighing about 20 g. were given about 5 mg. of micrococcin intravenously in 0·3 ml. of the serum-saline mixture described above on every second day for 10 days. The total doses given to the 4 mice were 27·3, 26·6, 26·6, 27·1 mg. respectively. No ill effects followed any of the injections, and these mice subsequently bred and produced young normally (see later). A second series of 6 mice was injected similarly except that the 5 doses were given on successive days. The total dose was 27·7 mg. in each case. None of these mice displayed any abnormality at any time. They were killed at intervals for histological and other observations.

Effect on weight.

Substantial doses of micrococcin had no perceptible effect on rate of increase in the weights of 6 mice, as compared with two control groups of animals (Fig. 1). The same was true of the mice described in the next paragraph.

Effect on reproduction.

As already mentioned, 4 female mice were given about 5 mg. suspended in 0·3 ml. of the serum-saline mixture every second day (total dose about 27 mg.). Control groups of mice were given the same volume of serum-saline mixture and of physiological saline. The weights of the control animals and of those

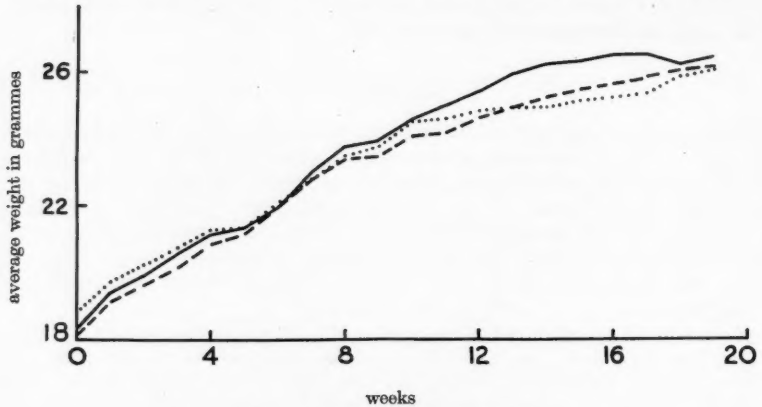

FIG. 1.—Average weight of 6 mice.

———— injected intravenously with 5 mg. of micrococcin in 0·3 ml. of 30 per cent serum per day for 5 days.
– – – – – „ „ with 0·3 ml. of 30 per cent serum.
. . . . . „ „ „ „ of normal saline.

injected with micrococcin increased in a parallel fashion. About 36 days after the termination of their injections both control and micrococcin-injected mice became pregnant. They all produced normal litters which developed normally.

The Distribution and Effects of Micrococcin in the Tissues following Intravenous Injection.

Distribution and persistence in the organs.

It was expected that injected particles of micrococcin would be abstracted from the blood-stream by the phagocytic cells of such organs as the liver and spleen, and that once removed they might remain in the cells for a long time provided that they did no harm. This proved to be the case, as shown below.

Zones of inhibition around crushed organs placed on assay plates.

The antibiotic could be detected by this crude method in liver and spleen for some weeks after the intravenous injection of 1 mg., though by 10 weeks little or no inhibition was obtained (Su and Florey, 1948, unpublished).

Quantitative estimation.

A weighed portion of the organ or tissue was minced with scissors and extracted with a known volume of alcohol. In the first few experiments the

micrococcin in the alcoholic extract was estimated microbiologically, but later a fluorimetric method was used, occasional checks showing agreement between the two methods.

The exact method of extraction was modified from time to time, but simple mincing of the tissue with 19 or 39 volumes (depending on the amount of tissue available) of alcohol, and leaving at room temperature for 2 hours or more with occasional shaking, followed by centrifuging, appeared to be quite satisfactory. When the whole carcass (usually minus certain organs) was to be assayed, the skin, tail and paws were removed and the rest was minced in a Waring " blendor " with not less than 80 ml. of alcohol (a smaller volume did not cover the blades). After standing for at least an hour the pulp was filtered through a folded filter-paper.

The microbiological and fluorimetric assay methods are described by Heatley, Kelly and Smith (1951). Care was taken that for the former, based on the solid medium dilution method of Su (1948), the alcohol concentration in the first tube did not exceed 1 per cent. For fluorimetric assays in the later work control extracts containing the same proportions of alcohol to tissue were sometimes prepared from a normal untreated animal, and corrections were made for non-specific fluorescence and for the quenching effect on micrococcin fluorescence of certain components of the extracts.

Quantitative results.

All the definitive experiments have been summarized in the histiogram (Fig. 2). The mice had been injected with different amounts of different samples of micrococcin at different times. The technique of extraction was not uniform throughout the series, and some assays were done microbiologically and some

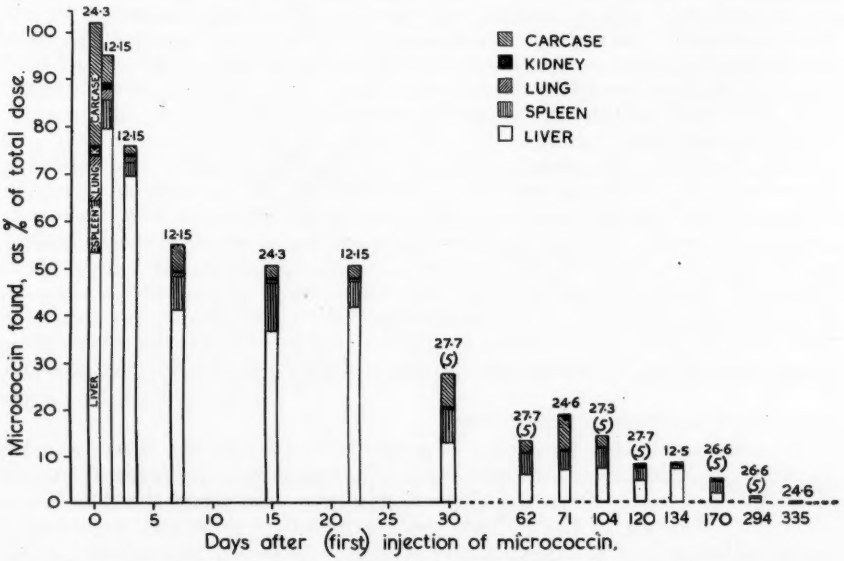

FIG. 2.—Distribution of micrococcin in the tissues at different times after injection.

Each column represents one mouse, the dose given (in mg.) being shown over the column. If the dose was divided the number of injections is shown in brackets. The amounts of micrococcin found are shown as percentages of the dose given.

fluorimetrically. The figure for total recovery does not include the amount of micrococcin (probably negligible) contained in certain pieces of tissue (lymph gland, bone, etc.) which were removed for histological examination and for which no correction could be made. The estimated accuracy of the assays for each individual mouse varies considerably, but for liver and spleen is believed to be within ± 5 per cent for most experiments. In the kidney and lung the amount of micrococcin was small; consequently the extracts could not be much diluted, if at all, and the errors due to non-specific fluorescence and quenching of the fluorescence of micrococcin are correspondingly more important. The degree of accuracy for these tissues is therefore much less. For the carcass the accuracy is slightly greater than for the lung and kidney. When only very low concentrations of micrococcin were present, more reliance was placed on microbiological than on fluorimetric assay.

Excretion.

Mice injected with micrococcin were kept in metabolism cages in which urine and faeces could largely be separated. Fluorimetric assay of the micrococcin could not be used as there was much non-specific fluorescence from these excreta, but microbiological assay of alcoholic extracts gave consistent results. Extracts of urine and faeces from a control mouse did not inhibit the test organism.

A mouse that had received 6·8 mg. of micrococcin in 0·5 ml. of fluid intravenously excreted 7·2, 1·8 and 0·04 per cent of the injected material in the first, second and third 24-hour periods respectively. The faeces contained 30 to 40 times as much as the urine, in which activity could scarcely be detected. When the mouse was killed on the fourth day the carcass contained 6·3 mg. of micrococcin, or 92·6 per cent of the dose given. Thus, within the limits of experimental error, the whole of the micrococcin disappearing from the body of the animal seemed to have been excreted.

The fact that micrococcin appeared in the faeces rather than the urine suggested that it might be excreted in the bile. It was not possible to test this rigorously, but gall bladders removed after 12 and 24 hours from mice which had received similar doses to the above contained a concentration which, assuming a bile-flow of 0·3–0·4 ml. per 24 hours, could account for the whole amount excreted. The concentration would be of the order of 1·5 mg. per ml.—about a thousand times that in a saturated watery solution. It was ascertained *in vitro* that even at room temperature a solution of bile salts in the concentration present in gall-bladder bile would dissolve at least 2 mg. of micrococcin per ml.

Macroscopic appearance of the organs.

In animals killed a few hours after the last injection the spleen and liver had a greyish appearance, and some degree of splenomegaly was present in animals killed up to 4 weeks from the time of injection. No other abnormality was observed. Portions of tissue from the organs to be examined were fixed in formol saline.

Histological Examination.

When paraffin sections were prepared the micrococcin was dissolved out by alcohol. The cells which had contained it were left with finely vacuolated

cytoplasm; these cells are referred to below as "micrococcin cells." The larger extracellular deposits left behind a cluster of vacuoles lying in a homogeneous eosinophil background. From the examination of paraffin sections alone, therefore, it was often possible to gain a general idea of the distribution of micrococcin in the tissues.

In frozen sections examined by ultra-violet light the particles of micrococcin fluoresced brilliantly.* When stained with haematoxylin the dim outline of the tissue could be seen as a blue shadow, with darker nuclei sometimes distinguishable. When the Woods glass filter was removed the general architecture of the tissue was clearly seen, while the fluorescent particles of micrococcin could still be picked out, and this method was used for the systematic examination of all sections and for photography. When a more detailed examination of the tissue was required the position of the fluorescent particle was marked with a marker eye-piece and the ultra-violet light replaced by white light.

Liver.

Large amounts of micrococcin were present in this organ a few hours after injection. Some of it had already been phagocytosed by the Kupffer cells, but the larger amount had been deposited in indefinite masses in the sinusoids or had become adherent to the endothelial lining of blood vessels (Fig. 3). At the end of 24 hours many Kupffer cells had become swollen, with, in paraffin sections, abundant vacuolated cytoplasm which pushed the nucleus to one side. Some of these cells had collected together in small groups and the outlines of the individual cells had become blurred. A few macrophages near the adventitia of vessels contained particles of micrococcin.

A feature of interest in the sections examined after the first 3 days was an apparent re-distribution of the particulate material within the organ. The groups of "micrococcin cells" already mentioned appeared to increase in volume and to acquire a larger amount of micrococcin. Multinucleated giant cells (Fig. 9) had been formed, and at the same time the number of single Kupffer cells which contained the substance had decreased.

In sections examined 8 days after a single injection most of the extracellular micrococcin had disappeared, and it was evident that the total concentration in the tissue had fallen considerably. The gradual decrease in intracellular as well as extracellular micrococcin continued, and by the 170th day it had disappeared from single cells. It could still be seen in the larger deposits on the 335th day, but the fluorescence had become very faint.

Fig. 4, 5, 6, 7 and 8 illustrate the changes in distribution and in concentration which have been described.

Spleen.

The concentration of micrococcin during the first few hours after injection was as high in the spleen as in the liver. The greatest concentration was in the pulp at the periphery of the Malpighian bodies, but some particles were scattered through the rest of the pulp. Some had been phagocytosed, but mostly they were in indefinite masses in the sinusoids (Fig. 10). By 24 hours a considerable proliferation of cells had occurred in the Malpighian bodies, causing them to

* We are indebted to Dr. J. R. Baker for advice on ultra-violet fluorescence microscopy.

spread outwards and form apparently continuous masses of cells in which many mitotic figures could be seen (Fig. 11). These cells had a large open nucleus and a moderate amount of basophilic cytoplasm. Plasma cells were also present. This activity gradually declined until by the 8th day discrete Malpighian bodies were again general, and there was no evidence of increased cell division (Fig. 12). During the period of hyperplasia the proliferating cells invaded the pulp and obscured the micrococcin particles so that their distribution was more difficult to follow (Fig. 13). With the subsidence of proliferation the micrococcin was found to be in its original site. By this time, however, all the micrococcin was intracellular, and could be identified in single cells, groups of cells and giant cells scattered throughout the pulp. A few particles had passed into the Malpighian bodies. By the 20th day the Malpighian bodies had returned to their normal size and remained in this state. No increase in reticulin or collagen formation was observed.

A similar though much smaller amount of hyperplasia was observed in a mouse which had received a control dose of serum-saline mixture without micrococcin.

Between the 20th and the 335th days there was a gradual falling off in the brightness of the fluorescence of the micrococcin deposits. The accumulation of the particles into larger aggregates such as was observed in the liver was also seen in the spleen.

Lungs.

Three hours after injection the amount of micrococcin present in the lungs was comparable to that in the liver at this time. Most of the particles had been deposited in masses in the capillaries of the alveolar walls, some were adherent to the endothelium of the larger vessels, and the rest had formed solid " micrococcin thrombi " in some of the smaller vessels (Fig. 14). The concentration of the substance in the lungs fell rapidly, and at the end of the first 24 hours micrococcin could only be seen in " thrombi," and in a few macrophages in the peribronchial and perivascular lymphatics in the hilar region of the lung (Fig. 15). The thrombi were gradually phagocytosed by macrophages, and this process was complete at the end of 8 days (Fig. 16). Micrococcin could still be detected in the resulting intravascular cell groups at the end of 230 days. No other histological changes were seen in any of the lungs examined.

Kidneys.

Three hours after injection micrococcin was found in the vessels and glomerular loops of the cortex (Fig. 17). The substance had disappeared by the end of 24 hours, and the presence of a few macrophages containing particles in the capsular lymphatics suggested that some had been removed *via* the lymphatic channels.

Bone marrow.

Paraffin sections of decalcified femur were examined, and compared with similar preparations from control animals. The marrow did not differ in architecture or in cellularity from that of the control mice. No " micrococcin

cells " could be identified in these preparations, so marrow smears were also examined.

Mice were killed 12, 24, 48 hours, 8 and 170 days after an intravenous injection of 5 mg. of micrococcin. After fixation marrow was scraped from the femur, smeared on a slide and mounted in gelatine. By ultra-violet light a moderate number of fluorescent particles, apparently intracellular, were seen in the earlier preparations, and a few weakly fluorescent particles at 170 days after injection. No fluorescent material was seen in a control smear from a normal mouse.

Other organs.

No histological abnormality was detected in the gut, adrenal or thymus, and " micrococcin cells " could not be identified in any of these tissues.

The effect on the blood counts.

Although experiments in the mouse did not show any great concentration of micrococcin in the phagocytic cells of the bone marrow after intravenous injection, it was important to know whether it had any effect on the production or maturation of blood cells. Rats were chosen for these observations.

Methods.—On four different days during the week before the first injection total red and white cell counts, and differential white cell counts, were made on each of three female white rats of approximately 260 g. weight. On the 8th day 39·4 mg. of micrococcin suspended in rat serum and saline were given intravenously to each animal. The blood counts were then repeated at intervals of 3 to 4 days until the 24th day, when a second injection of 40·2 mg. of micrococcin was given. Blood counts were continued at intervals until the rats were killed on the 48th, 68th and 168th days respectively.

Results.—The injections of micrococcin did not appear to affect the general condition of the animals in any way.

The counts obtained in the first four examinations gave some indication of the normal for the rats used. The erythrocyte count of normal rats as estimated by different workers lies between 6·60 and 9·53 millions, and the total leucocyte count between 6000 and 20,000 (Cameron and Watson, 1949; Wills and Mehta, 1930–31; Thewlis and Meyer, 1942; Wintrobe, Schumacker and Schmidt, 1936).

Fig. 18 shows in graphic form the behaviour of the cells in one rat. The other two animals presented a very similar picture. Following the injections there was a rise in the total number of leucocytes, which appeared to be primarily due to an increase in the number of lymphocytes and monocytes, though in two out of the three animals there was a small increase in polymorphonuclears after the second injection. No abnormality was detected in either red or white cells in any of the blood films examined.

Local Effects of Micrococcin.

Reaction of subcutaneous tissue.

In order to observe the reaction of subcutaneous tissue to a relatively large amount of micrococcin, 1 mg. suspended in 0·2 ml. of sterile distilled water was injected subcutaneously into each of 3 mice. These animals were killed 7, 14, and 21 days after the injection. The resulting local lesion was a firm subcutaneous

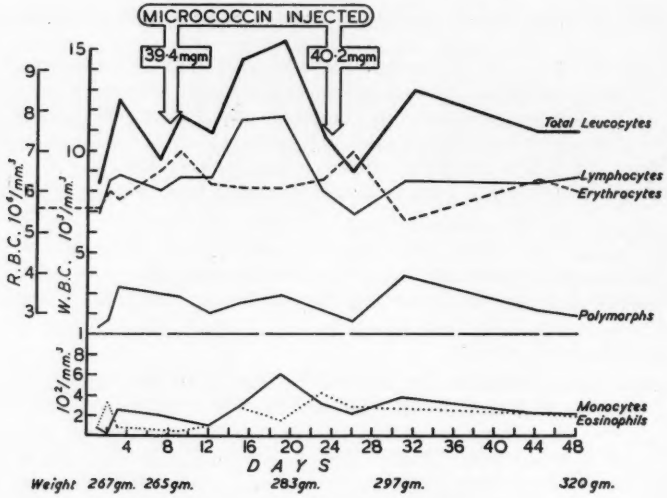

FIG. 18.—Graph of blood counts of rat " A."

nodule. Paraffin sections showed it to consist of large foamy macrophages, among which the remains of a few polymorphs could be detected. In the earliest nodule there was a central mass of eosinophilic material. In all the sections there was a narrow zone of young fibroblasts around the zone of foamy macrophages (Fig. 19).

Vascular reactions in the mouse's ear.

An irritant in the tissues will cause proliferation of blood and lymphatic vessels in its neighbourhood, the latter being demonstrable with some elegance in the mouse's ear (e.g., Pullinger and Florey, 1937). To ascertain whether micrococcin stimulated the proliferation of vessels small deposits were injected subcutaneously into the ears of anaesthetized mice by a micropipette, and later the lymphatic and blood vessels were injected.

Lymphatic vessels.

Fig. 20 shows that new fine lymphatics had formed around a small deposit of micrococcin which had been present for 15½ weeks. This observation was repeated frequently on mice bearing micrococcin for a shorter or a longer time, and left no doubt that the presence of micrococcin in the tissues caused a proliferation of neighbouring lymphatics.

Blood vessels.

Observations with the binocular dissecting microscope of the living ear illuminated by a total reflection rod (as for lymphatic injections) did not show any dilated or new vessels in the neighbourhood of the micrococcin deposit in any of the

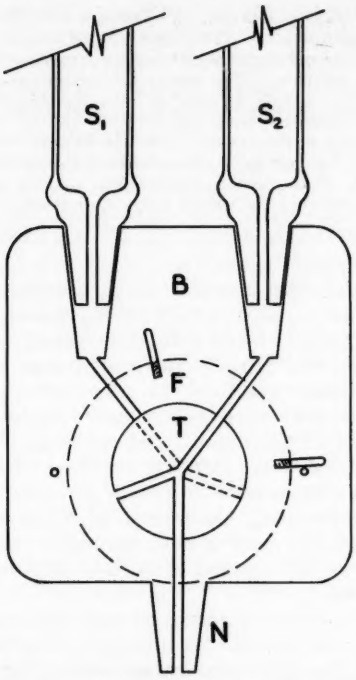

FIG. 21.—B is the body of the apparatus, T the key of the tap and F the flange of the tap. The lower exit (N) is made in the form of a cone to fit into a No. 16 needle. The two upper exits have conical holes into which fit the barrels of two glass tuberculin syringes (S_1 and S_2). Either syringe can be put in communication with the needle by turning the tap.

mice examined. However, new vessels might not have been seen if they were not filled with blood, so permanent preparations were made by injecting the vessels with a mixture of carbon and gelatine and subsequently clearing and mounting the ear. Only in an occasional specimen could an increase in the number of fine blood vessels be demonstrated.

It is interesting that the lymphatic vessels have proliferated more readily in response to a bland foreign body than have the blood vessels, which in the above experiments were apparently rarely stimulated to proliferate.

The effect of injection into the subarachnoid space.

Technique for intracisternal injection in the rabbit.—The withdrawal of cerebrospinal fluid and intrathecal injection of test substances in rabbits was facilitated by the use of a simple piece of apparatus made for the purpose. This consists of a tap made of Perspex (Fig. 21), into which the barrels of 2 tuberculin syringes are fitted. Into the upper ends of the syringe barrels brass adaptors are fitted, and on to these go two lengths of Portex tubing (Portex is a transparent plastic). These tubes are fitted to the nozzles of a pair of syringes mounted on a small board (Fig. 22). By manipulating the plungers of these syringes alterations can be made by an assistant of the pressure in the barrels of the syringes attached to the tap without interference with the operator. To use the apparatus, the

rabbit is anaesthetized and its neck shaved. The neck is then flexed and held so in a clamp (Fig. 23 and 24) attached to a board. The degree of flexion can be momentarily increased at the time of insertion of the needle without causing asphyxia of the animal. Syringe S_1 is first filled with the test solution. The tap is rotated to put syringe S_2 into communication with the needle. The needle is inserted into the cisterna magna and held quite still by the operator. His assistant then aspirates 0·5 ml. of c.s.f. into syringe S_2, turns the tap, and injects up to 1 ml. of the test solution. A drop or two of fluid is again aspirated by lessening the pressure in the syringe S_1 to make sure that the needle point has remained in the cistern all the time. With a little practice clear c.s.f. is obtained on most occasions, and if innocuous fluids are injected the rabbit recovers normally from the anaesthetic.

Results of injection.

A suspension of micrococcin in either rabbit cerebrospinal fluid or a mixture of saline and rabbit serum, approximately 20 mg. per ml., was injected into the cisterna magna of 10 rabbits. In no animal, following recovery from the anaesthetic, was there any sign of damage to the central nervous system.

Twenty-four hours after injection the c.s.f. cell count was approximately 1000 per c.mm. and the cells were mostly polymorphs. Three days later the cell count had fallen to half this figure, and polymorphs and lymphocytes were present in about equal numbers. After from 10 to 16 days the cell count had returned to normal. Amounts of micrococcin so small as to be at the limit of detection were found in the fluid, but after 1 month it could not be detected in the fluid by fluorimetric assay. Several of the rabbits were kept under observation for 5 months, and at no time did they show any sign of cerebral or spinal cord damage.

Paraffin and frozen sections of the brain and spinal cord of 3 rabbits killed 22, 31 and 46 days after the intrathecal injection of micrococcin were examined. Occasional plaques of foamy, micrococcin-containing macrophages adhered to the pia mater, but no other histological abnormality was seen.

Observations in transparent chambers in the rabbit's ear.

Observations were made in modified Sandison-Clark chambers on cellular and vascular reactions to micrococcin inserted into the chamber.

The first observations were made in a type of chamber previously described (Ebert, Florey and Pullinger, 1939), in which the table, over which a thin layer of tissue grows, is pierced by a plug 2 mm. in diameter. This plug can be removed and replaced, so that materials can be inserted into the chamber.

In the experiment with this chamber the blood vessels and accompanying tissue cells grew rather slowly, and had only partially covered the central table before the plug was removed. The plug was withdrawn without apparently causing trauma to the tissue or damage to the blood vessels ; some moist debris immediately over it was removed from the chamber, and the hole from which it had been withdrawn was filled with a fine suspension of micrococcin prepared by grinding amorphous material in saline. As the plug was replaced, some of the micrococcin was forced into the chamber. At the end of the operation micrococcin was present in considerable quantity in some parts of the chamber, and there was a continuous layer over the plug and in its immediate neighbourhood. The reaction to the trauma of the manoeuvre was slight.

As is usual in chambers in which new tissue is being formed, there were many macrophages in advance of and around the growing blood vessels. These cells

readily ingested the micrococcin, which apparently had no harmful effect on them, for they were still able to segregate vital new red injected intravenously. The blood vessels grew among the masses of micrococcin in a way indistinguishable from that usually observed in growing tissue. The vascular endothelium appeared to be unaffected by the micrococcin, for the leucocytes did not adhere to the vessel walls in its neighbourhood.

This chamber was observed for 137 days, throughout which time micrococcin was to be seen in many of the macrophages present. At the periphery of the chamber the macrophages disappeared to a large extent, leaving only a few along the outside of the blood vessels, and at about 100 days no micrococcin was discernible in this area. The blood vessels only penetrated into half the compact mass over the central plug, in which area the antibiotic, which was still present in considerable amount, had become somewhat yellowish. Some of these appearances are illustrated in Fig. 25 and 26.

Similar observations were made in a chamber so designed that the mica coverslip or "lid" was removable. When the central table area was covered by vascular fibrous tissue the lid was lifted off and a new mica slip was immediately applied. Unfortunately it was screwed down too tightly, and the circulation over the table area was stopped for long enough to kill the tissue. Seven days later the lid was again removed, a drop of micrococcin suspension was applied over the table area, and a new lid was put on. The dead tissue was revascularized by the ingrowth of new vessels from the periphery. Precisely the same phenomena were observed as in the first chamber. There was no evidence that the micrococcin ingested by the macrophages of the newly developing tissue damaged them, nor did uningested micrococcin appear to influence the growing vascular endothelium.

Experiments were also done with very finely particulate suspensions of micrococcin made as described at the beginning of this paper, placed in one fully grown chamber by removal of the lid, and in another through a hole closed by a silver pin. The macrophages ingested the material and some became full of particles, but, as before, they became fewer with the passage of time. It was found possible to illuminate the chambers by long wave ultra-violet light without damaging the tissue, and thereby to see the bright fluorescence of the micrococcin in the cells (Fig. 27 *a* and *b*).

All these experiments agreed in showing that micrococcin was ingested by macrophages and retained by them for many months. It caused no discernible damage to them, to delicate growing or to more mature tissues.

DISCUSSION.

The various methods which have been used demonstrate that the almost insoluble antibiotic micrococcin acted as a bland foreign body when introduced into the body. Its presence in macrophages appeared to be harmless, while around masses of it introduced subcutaneously a mild reaction occurred such as can be seen near foreign bodies, for example catgut. It remained for a considerable time in cells of the macrophage system and was slowly eliminated from the body, probably principally by way of the bile. That it retained its antibacterial activity in the body was shown by the area of inhibition around a crushed organ placed on an agar plate seeded with *Staphylococcus* and incubated.

Micrococcin appeared therefore to have properties which made its behaviour in experimental tuberculosis worth investigating. The results, to be reported later (Markham, Wells, Heatley and Florey, 1951), were disappointing, but it is suggested that the experimental methods described in this paper may be worth following when investigating other insoluble antibiotics with greater power to inhibit the growth of the tubercle bacillus than is possessed by micrococcin. It is also conceivable that intracellular infections of the reticulo-endothelial system might be treated by the administration of particulate suspensions of a drug which is known to have *in vitro* activity against the infecting organism.

SUMMARY.

1. A method is described for making a stable fine suspension of micrococcin suitable for intravenous injection.

2. Micrococcin injected intravenously is removed from the blood stream by phagocytic cells, in which it may remain for months without causing apparent damage.

3. Micrococcin is slowly eliminated from the body, probably mostly by way of the bile.

4. Micrococcin can be estimated in the tissues either microbiologically or by its fluorescence.

5. Micrococcin retains its antibacterial activity in the body.

6. The behaviour of micrococcin introduced into macrophages has been watched in transparent chambers in the rabbit's ear.

7. All experiments showed that micrococcin behaved in the tissues like a bland foreign body.

ACKNOWLEDGMENTS.

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EXPLANATION OF PLATES.

FIG. 3.—Liver of a mouse killed 3½ hours after intravenous injection of 25 mg. of micrococcin. Micrococcin is filling the sinusoids and is adherent to the vessel walls.

Ultra-violet light. ⅔-in. objective.

FIG. 4.—Liver of a mouse killed 24 hours after intravenous injection of 12·5 mg. of micrococcin. Most of the free micrococcin has been cleared from the sinusoids and is now in the Kupffer cells.

Ultra-violet light. ⅔-in. objective.

FIG. 5.—Liver of a mouse killed 3 days after intravenous injection of 12·5 mg. of micrococcin. Some decrease in the concentration has occurred.

Ultra-violet light. ⅔-in. objective.

FIG. 6.—Liver of a mouse killed 8 days after an intravenous injection of 12·5 mg. of micrococcin. The total concentration of micrococcin has fallen considerably. Many of the smaller deposits have disappeared. Micrococcin-containing cells can be seen about the blood vessels.

Ultra-violet light. ⅔-in. objective.

FIG. 7.—Liver of a mouse killed 22 days after an intravenous injection of 12·5 mg. of micrococcin. Deposits in individual Kupffer cells have almost entirely disappeared, and there has been an increase in the size of the larger deposits.

Ultra-violet light. ⅔-in. objective.

FIG. 8.—Liver of a mouse killed 134 days after an intravenous injection of 12·5 mg. of micrococcin. Only a few deposits remain, and some of these are becoming faint.

Ultra-violet light. ⅔-in. objective.

FIG. 9.—A " micrococcin giant cell " in the liver of a mouse killed 62 days after intravenous injections of micrococcin suspension (total dose 27·5 mg.).

Haematoxylin and eosin. $\frac{1}{12}$-in. objective.

FIG. 10.—Spleen of a mouse killed 3·5 hours after an intravenous injection of 25 mg. of micrococcin. The heaviest concentration is at the periphery of the Malpighian bodies, but large irregular deposits are also present in the pulp.

Ultra-violet light. ⅔-in. objective.

FIG. 11.—The same spleen as that shown in Fig. 13. The mouse was killed 24 hours after an injection of 12·5 mg. of micrococcin. There is marked hyperplasia of the Malpighian bodies.

Haematoxylin and eosin.

FIG. 12.—Spleen of a mouse killed 8 days after an intravenous injection of 12·5 mg. of micrococcin. There is less hyperplasia than in that shown in Fig. 11. Discrete Malpighian bodies are present.

Haematoxylin and eosin.

FIG. 13.—Spleen of a mouse killed 24 hours after an intravenous injection of 12·5 mg. of micrococcin. The deposits are separated and are obscured by the enlarged Malpighian bodies.

Ultra-violet light. ⅔-in. objective.

FIG. 14.—Lung of a mouse killed 3 hours after an injection of 25 mg. of micrococcin. This animal was sick when it was killed. Note the micrococcin in the alveolar walls and the presence of several " micrococcin thrombi."

Ultra-violet light. ⅔-in. objective.

FIG. 15.—Lung of a mouse killed 24 hours after injection with 12·5 mg. of micrococcin. Note the presence of micrococcin particles in the hilar lymphatics.

Ultra-violet light. ⅔-in. objective.

FIG. 16.—Micrococcin-filled macrophages at the site of a " micrococcin thrombus " in the lung. Animal killed 8 days after injection with 12·5 mg. of micrococcin.

Paraffin section. Haematoxylin and eosin. $\frac{1}{12}$-in. objective.

FIG. 17.—Kidney of a mouse killed 3·5 hours after an injection of 25 mg. of micrococcin. Micrococcin can be seen in the cortical glomeruli and capillaries.

Ultra-violet light. ⅔-in. objective.

FIG. 19.—A high-power view of a part of a subcutaneous nodule showing the character and arrangement of the foamy cells.

Paraffin section. van Gieson. $\frac{1}{6}$-in. objective.

FIG. 20.—Ear of mouse. Lymphatics injected with carbon suspension 109 days after the subcutaneous injection of a very small drop of a suspension of micrococcin. The lymphatics have proliferated around the deposit of micrococcin.

× 32 approx.

FIG. 22.—General view of the two-way tap for a pair of remote controlled syringes for cisternal puncture in the rabbit.

FIG. 23.—Board for holding a rabbit for cisternal puncture. The animal's body is kept in place by sand-bags, and its head is securely held in a rubber-padded clamp so as to maintain full flexion of the neck.

FIG. 24.—View of the two-way tap and a pair of syringes in use.

(Note : for the sake of clarity the sterile towel over the body of the rabbit was omitted when this photograph was taken.)

FIG. 25.—Macrophages containing micrococcin lying between blood vessels : 4 days after insertion of the antibiotic into the ear chamber.

$\frac{2}{3}$-in. objective.

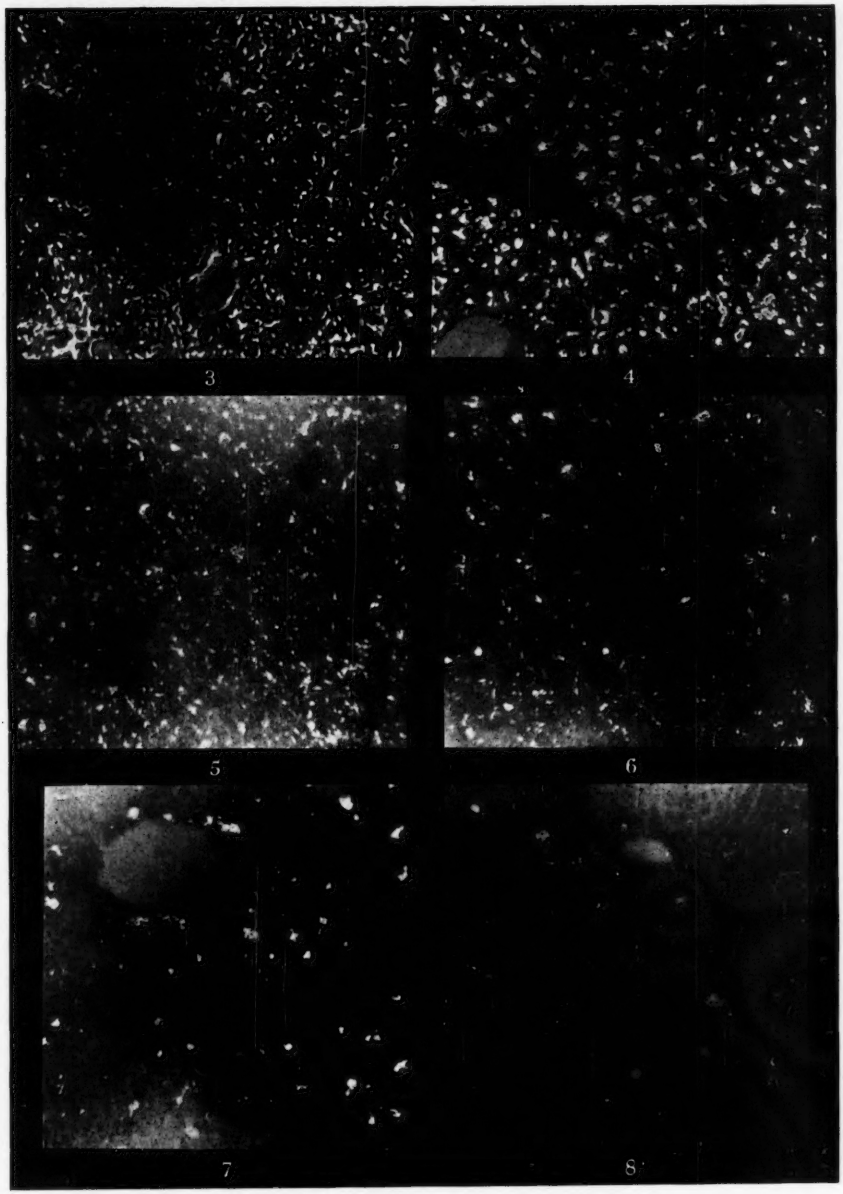
FIG. 26.—The same field as Fig. 25 : 36 days after insertion.

$\frac{2}{3}$-in. objective.

FIG. 27.—Macrophages full of micrococcin.

(*a*) Photograph taken with visible light with a green filter ;
(*b*) „ „ „ ultra-violet light to show fluorescence of micrococcin.

$\frac{1}{6}$-in. objective.



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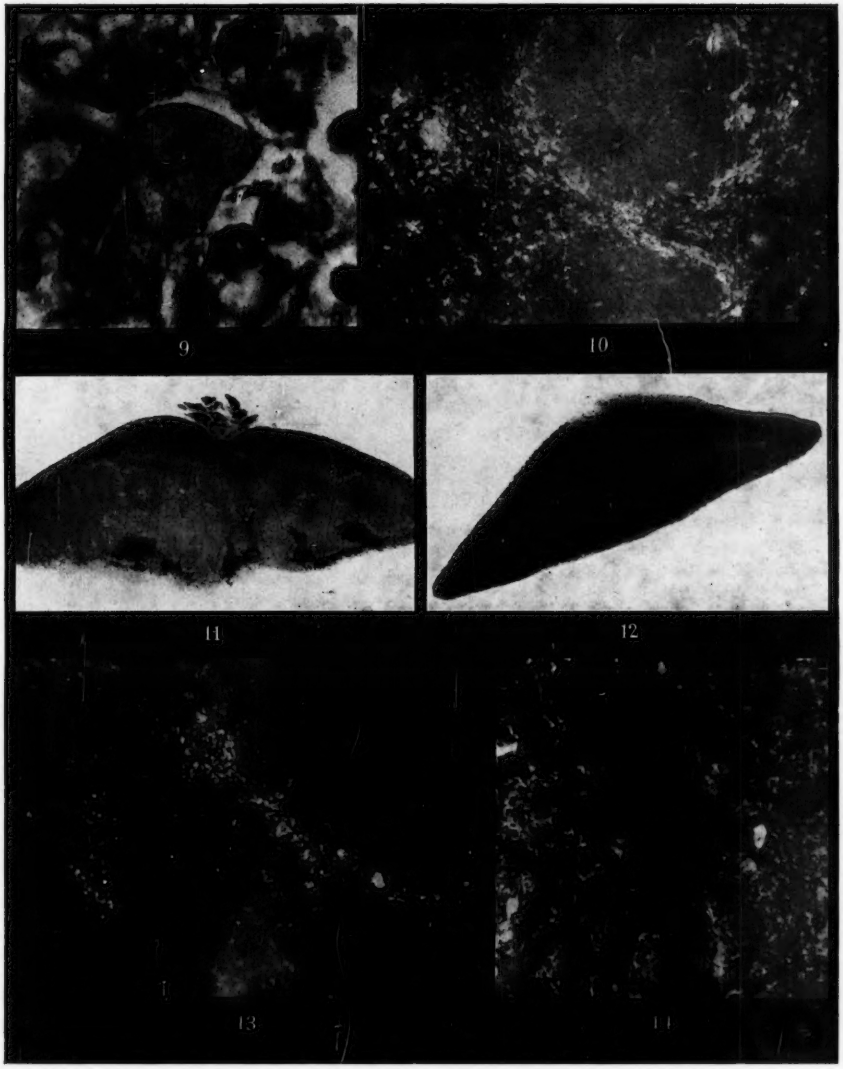

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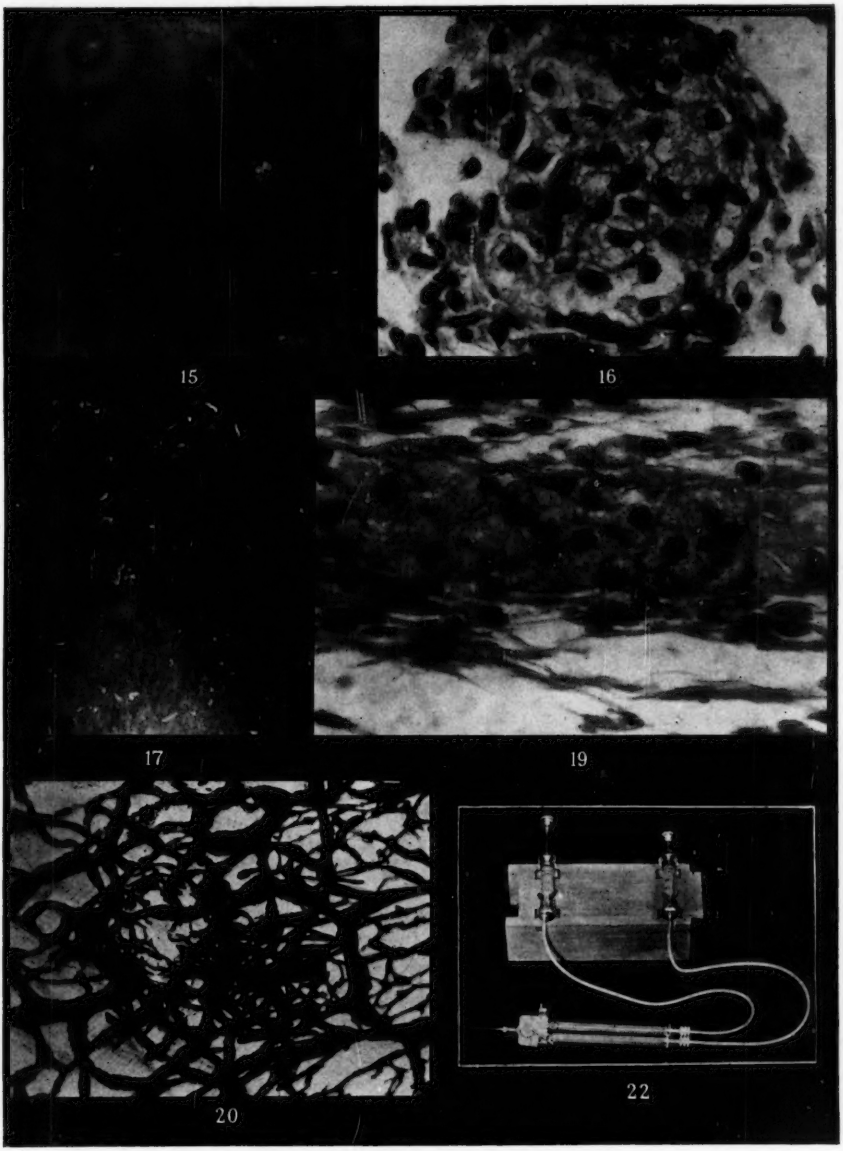

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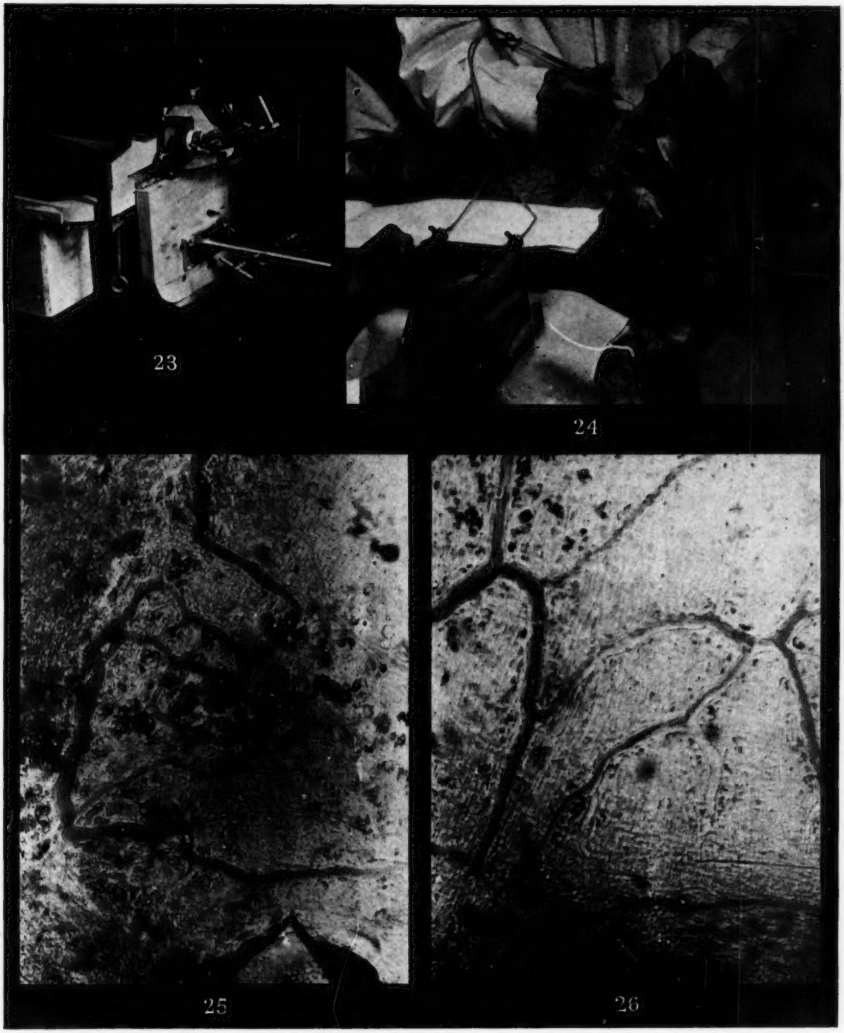

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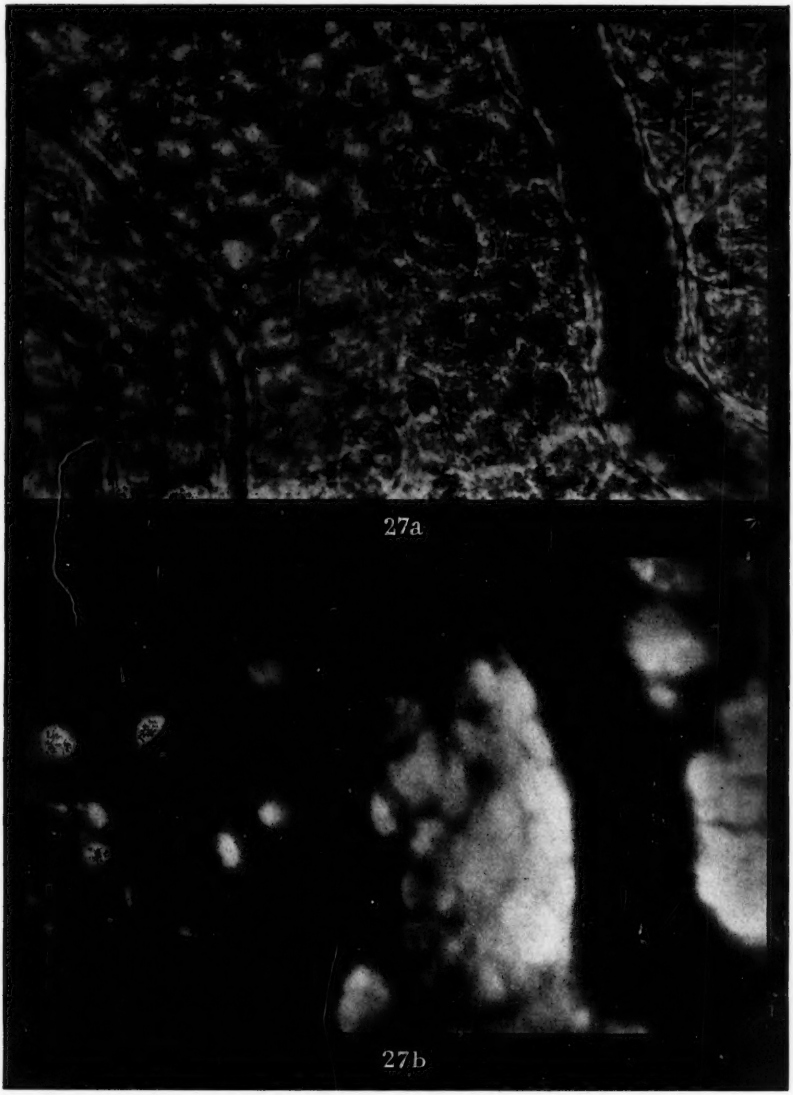

27a

27b

EXPERIMENTAL STAPHYLOCOCCAL INFECTIONS IN MICE.

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THE ability of staphylococci to infect animals is related to their ability to clot the animals' plasma. Wilson Smith and Hale (1944, 1947) showed that the coagulase test depends on the interaction of the staphylococcal coagulase with a labile factor in the animals' plasma, which they termed activator substance. As a result of the interaction a substance is produced which converts fibrinogen into fibrin. This may be compared with the prothrombin-thrombokinase mechanism of normal blood-clotting. Some animal strains appear to lack activator substance in their plasma, and such plasma fails to give a coagulase test with a known coagulase-positive staphylococcus. Animals with such a deficient plasma were found to be relatively insusceptible to staphylococcal infection. Staphylococci injected into a susceptible animal appeared to be partially protected from phagocytosis by the fibrinous clot forming around them. In an animal lacking activator substance the staphylococci were rapidly phagocytosed. In rabbits, which have a coagulable plasma, coagulase-positive strains are pathogenic and coagulase-negative strains are not (Cruickshank, 1937).

Mice are less susceptible to staphylococci than rabbits, and this is said to be due to the lack of activator substance in the plasma (Wilson Smith and Hale, 1947). Wilson Smith and Hale used intraperitoneal injections of staphylococci, and the resistance of mice to the staphylococcus by this route is well known. In the present study it was also found that staphylococci instilled intranasally suspended in saline, broth or serum fail to infect mice. However, in the course of some experiments it was noticed that mouse plasma was clotted by coagulase-positive staphylococci. A similar observation was recorded by Field and Smith (1945), who used the slide coagulase technique in an attempt to correlate the origin of the staphylococcus with the types of plasma it would clot. They found that staphylococci isolated from food, human, ox, sheep, dog, horse, rabbit and goose would clot human, bovine, mouse, dog, swine, rabbit and cat plasma.

METHODS.

The bulk of the work was carried out on Swiss White No. 1 mice supplied by one dealer. They were used when about 12 weeks old and weighed, on the average, 20 grammes. Later, through the kindness of Prof. A. Haddow, two pure strains of mice were obtained. These were (1) C57, a black mouse, and (2) ABC, a brown mouse closely resembling in appearance strain CBA. These two strains were tested in the same way as the Swiss No. 1 mice. The mice were examined *post mortem* by the aseptic technique of Himmelweit (1944).

Plasma was obtained from normal mice as follows : Under chloroform anaesthesia the thorax was opened aseptically and 0·5 ml. of sterile 3·8 per cent sodium citrate introduced. The apex of the heart was cut with fine scissors and the resultant flow of blood mixed with the citrate to prevent clotting. More blood was expressed from the liver and viscera by gentle pressure with a swab. The citrated blood was then removed with a wide-bore Pasteur pipette. This method yielded about 2·0 ml. of blood from a 20-g. mouse (Himmelweit, personal communication).

Slide technique.

Staphylococci grown on plain agar for 18 hours were suspended in two loopfuls of distilled water and mixed with one loopful of undiluted plasma by rocking.

Tube method.

To 0·5 ml. of human plasma diluted 1 : 10, 0·1 ml., of 18-hour plain broth culture was added. The mixture was incubated at 37° C. in a waterbath and read at 30-minute intervals. With mouse plasma two tubes were set up. One was kept at 37° C. for 3 hours and the other at room temperature overnight. Room temperature incubation was used in order to detect activator substance in low concentration which is said to be slowly destroyed at 37° C.

Mouse pathogenicity.

Mice received 0·1 ml. (approximately 40 million organisms) of an overnight plain broth culture injected into a tail vein. Six mice were injected with each strain of staphylococcus.

RESULTS.

Twenty strains of staphylococci were examined ; they consisted of 12 coagulase-positive strains and 8 coagulase-negative strains as tested against human plasma. With the exception of one strain of staphylococcus, all the coagulase-positive staphylococci gave positive results with mouse and human plasma, tested both on the slide and in the tube. The exceptional strain, which was negative with mouse plasma, was re-tested against 8 different samples of mouse plasma. Two samples were positive after standing at room temperature overnight ; none was positive at 37° C.

Following injection, any mouse surviving on the 14th day was killed and examined for macroscopic lesions and cultured for staphylococci. All mice dying before the 14th day had macroscopic lesions in the kidneys, and the abscesses yielded a pure growth of *Staph. aureus.*

In the 11 surviving mice, 5 had visible abscesses in the kidneys, and these and 3 of the others had positive cultures from their kidneys.

The histogram (Fig. 1) was constructed from the data obtained, and shows that the highest mortality occurs in the 3rd, 4th and 5th days after inoculation.

In animals dying during the experiment the characteristic finding was the multiple kidney abscesses. These varied considerably in size, the largest being 2–3 mm. in diameter. The lungs and liver did not show any macroscopic lesions in any case. One spleen contained a small abscess. Another spleen was adherent to a kidney abscess ; spleens from the remainder were sometimes enlarged but

never showed naked-eye lesions. Cultures from the various organs were taken. The lungs, liver and heart muscle were sterile. *Staph. aureus* was cultured from three spleens out of eight, including the spleen containing a visible abscess. Various organs were examined histologically with the following results :

Spleen : Apart from the one abscess mentioned above, histological examination showed no abscess formation in 6 spleens.

Liver : Four livers examined showed no abnormality.

Lungs : Nine out of 12 lungs showed patchy congestion with oedema.

Heart muscle : Three hearts were examined and no lesion was detected.

Kidneys : In all, 20 kidneys were examined from 61 animals which died. The general picture was one of multiple medullary and cortical abscesses. In the

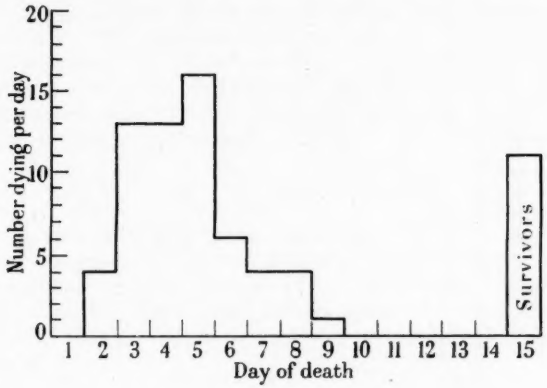

FIG. 1.

remains of some of the tubules solid masses of Gram-positive cocci were seen. Some of the tubules contained pus cells.

The effects of dilution of the broth culture were next investigated. A 1 in 10 dilution (dose 4 million organisms) produced a mortality rate of between 40 and 50 per cent of the mice injected, with a peak of mortality at the 7th and 8th day. A 1 in 100 dilution (dose 400,000 organisms) was followed by no deaths in 14 days.

The remaining 8 strains of staphylococci were coagulase-negative when tested against mouse and human plasma, both by tube and slide methods. These strains were injected into mice, each strain being tested in 6 animals. To summarize the findings, 5 out of 48 mice died. Two of these were found dead and partly eaten with no lesions in their kidneys at post-mortem examination. In the other 3 mice, lesions suggestive of staphylococcal infection were not found. The deaths occurred on 7th, 8th, 10th, 11th and 12th days, which is later than the usual time of deaths occurring from acute staphylococcal infection. Twelve mice gave a positive culture from the kidneys at post-mortem examination.

Effects on different strains of mice.

Experiments were carried out to determine whether this susceptibility to staphylococcus was shared by other strains of mice. Seven coagulase-positive

staphylococci gave a positive coagulase test with plasma from the ABC and C57 mice and 7 negative strains failed to clot plasma from these mice. Each strain of staphylococcus was injected into the different strains of mice on the same day.

The Swiss mice were inoculated with the 7 coagulase-positive strains, while the ABC strain was inoculated with 6 coagulase-positive staphylococci and the C57 strain with 5 coagulase-positive staphylococci. In general it may be said that the results with the two strains of coloured mice are similar to those obtained with the white Swiss No. 1 strain.

A polygram (Fig. 2) has been constructed from the data obtained to show the results graphically. It was noticed that the C57 strain showed a larger percentage of survivors than the ABC and Swiss No. 1. Comparing the death rates

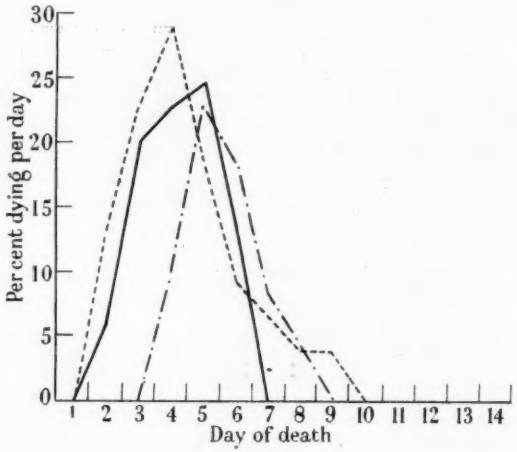

FIG. 2.

at 14 days in the three strains it was found that C57 is significantly more resistant than ABC or Swiss No. 1. There was no significant difference between ABC and Swiss No. 1.

The limited number of mice of strains C57 and ABC precluded extensive investigation of the effects of coagulase-negative staphylococci, but 2 strains of staphylococci (28, 29) were injected into the 3 strains of mice. Similar results were obtained to those recorded with the Swiss mice previously. No mice died within 14 days ; 5 out of 36 mice showed positive cultures at post-mortem examination.

In order to determine the site of the earliest lesion, Swiss No. 1 mice were injected with 0·1 ml. of a coagulase-positive staphylococcus and killed at intervals. The kidneys were removed and examined histologically. In kidneys removed from mice at 42 hours, before the appearance of abscesses, organisms could be seen in damaged glomeruli and both around and inside the lumen of the tubules. The organisms were surrounded by leucocytes.

In order to determine the effect of mouse passage on the pathogenicity of staphylococci for mice, 6 strains of coagulase-positive staphylococci, after five

passages through mice, were grown in broth and then injected intraperitoneally into white mice. Doses of up to 0·4 ml. were given by this route without causing death in 14 days. At the end of this time the mice were killed and examined and none of them were found to have kidney abscesses. Then the spun deposit of 0·2 ml. of broth culture of these passaged strains was suspended in human serum and introduced intranasally in mice without effect.

DISCUSSION.

Mice are extremely resistant to staphylococci by the intraperitoneal and intranasal routes, but they are susceptible to intravenous inoculation of coagulase-positive staphylococci. When they are inoculated by this route the characteristic lesion is in the kidney, and is very similar to that produced in rabbits by the same procedure. This experimental procedure is of value as a cheap and efficient method of testing staphylococci for pathogenicity. It might also prove of value in testing the efficacy of antibiotics against pathogenic staphylococci *in vivo*. It confirms the observation of Wilson Smith and Hale that the pathogenicity of staphylococci is related to the ability to clot the test animal's plasma.

SUMMARY.

Mouse plasma may be used successfully in the staphylocoagulase test. Mice are susceptible to coagulase-positive staphylococci injected intravenously and are resistant to coagulase-negative strains. The characteristic finding is multiple abscesses in the kidneys. The earliest lesion appears to be in the glomeruli, with the tubules affected shortly afterwards. These results were obtained using three different strains of mice.

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